JOURNAL OF AGRICULTURAL RESEARCH

VOL. 57 WASHINGTON, D. C., SEPTEMBER 15, 1938 No. 6

FOLIAR DIAGNOSIS IN RELATION TO DEVELOPMENT AND FERTILIZER TREATMENT OF THE POTATO[1]

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INTRODUCTION

Foliar diagnosis is defined (*1, 6*) [2] as the chemical condition or state (chemism) of a leaf with respect to the dominant nutritive elements (entities) at the instant of sampling, and taken from a predetermined and suitable position. The annual foliar diagnosis of a plant consists, then, of a sequence of conditions or states with respect to the quantities of the dominant nutritive elements in the leaf, taken from a predetermined position, as revealed by analysis at different stages during the growth cycle from one year to another on the same soil in a given location.

It has been pointed out (*6*) that the keystone of the method of foliar diagnosis rests upon facts experimentally established [3] (*2, 5, 7*), which, briefly summarized, are as follows:

Plants growing on two experimental plots which are similarly nourished (as deduced from similar development and yields) are represented in general and with a sufficient approximation by similar foliar diagnoses; and, conversely, plants growing on two plots which are differently nourished (as deduced from different development and different yields) are represented by different foliar diagnoses.

The purpose of the work reported in this paper was to determine relation of the foliar diagnosis of potato plants grown on the vegetable fertility plots of the Pennsylvania Agricultural Experiment Station to the development of plants from duplicate (similarly fertilized) plots and to the fertilizer applied.

The point of departure of the method of foliar diagnosis from the methods of traditional agronomy is that it utilizes as an analytical expression of plant performance not the soil nor the fertilizer applied, but the chemical condition of the leaf at different stages in its life cycle.

MATERIALS AND METHODS

EXPERIMENTAL PLOTS

The potato plants used in the present investigation were grown on tiers 1, 2, 4, and 5 of the station vegetable-fertility plots which were laid out in 1916. A description of the experimental plan has been given by Mack (*3*). The plots [4] are 12 by 36.3 feet and are separated

[1] Received for publication January 18, 1938; issued September 1938. Paper No. 819 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

[2] Italic numbers in parentheses refer to Literature Cited, p. 414.

[3] THOMAS, WALTER. COMPOSITION OF LEAVES OF THE SAME PHYSIOLOGICAL AGE, AS DETERMINED PERIODICALLY, OF APPLE TREES GROWN IN METAL CYLINDERS AND SUBJECTED TO DIFFERENT TREATMENTS WITH NUTRIENT SALTS. Unpublished.

[4] To conserve space, where tier and plot are referred to together, two figures are used, the first indicating tier, the second, plot; as, 1-2—tier 1, plot 2.

by guard strips 6 feet wide; the fertilizer treatment of each plot extends to the middle of the guard strip. The area of the plots exclusive of guard strips is one-hundredth of an acre. The experimental crops consist of a 4-year rotation of cabbage, potatoes, tomatoes, and sweet corn. Rye and vetch are used as a cover crop. The fertilizers are applied in different combinations before the last harrowing and the manure (partly rotted horse manure) before the land is plowed. The variety of potato (*Solanum tuberosum* L.) is the Rural Russet. The tiers and plots examined in 1935 together with their fertilizer treatments are given in table 1.

TABLE 1.—*Tiers and plots examined and fertilizer treatment given, 1935*

Tier No.	Plot No.	Fertilizers applied [1]	Amount applied to plot	N, P_2O_5, and K_2O equivalents (pounds)	Symbol and ratio
			Pounds		
1	2	Sodium nitrate	4.0	0.6	N.
4	16				
1	3	Superphosphate	6.25	1.0	P.
4	15				
1	4	Potassium chloride	1.666	.8	K.
4	14				
1	6	Sodium nitrate	4.0	.6	NP.
4	12	Superphosphate	6.25	1.0	
1	7	Sodium nitrate	4.0	.6	NK.
4	11	Potassium chloride	1.666	.8	
1	8	Superphosphate	6.25	1.0	PK.
4	10	Potassium chloride	1.666	.8	
1	10	Sodium nitrate	4.0	.6	NPK, 6:10:8.
4	8	Superphosphate	6.25	1.0	
		Potassium chloride	1.666	.8	
1	15	Rotted horse manure	[2] 600	4.62 (N)	15:10:12.3.[3]
4	3			3.08 (P_2O_5)	
				3.78 (K_2O)	
		Sodium nitrate	6.0	.9	(1.5N) PK, 9:10:8.
2	4	Superphosphate	6.25	1.0	
5	4	Potassium chloride	1.666	.8	
		Sodium nitrate	4.0	.6	N(1.5P)K, 6:15:8.
2	8	Superphosphate	9.375	1.5	
5	8	Potassium chloride	1.666	.8	
		Sodium nitrate	4.0	.6	NP(1.5K), 6:10:12.
2	12	Superphosphate	6.25	1.0	
5	6	Potassium chloride	2.499	1.2	
		Sodium nitrate	2.0	.3	0.5(NPK), 3:5:4.
2	14	Superphosphate	3.125	.5	
5	4	Potassium chloride	.833	.4	
		Sodium nitrate	8.0	1.2	2(NPK), 12:20:16.
2	10	Superphosphate	13.5	2.0	
5	2	Potassium chloride	3.332	1.6	

[1] Fertilizers within braces were all applied to both plots given in previous column.
[2] Containing 65.7 percent of moisture.
[3] Ratio is on a basis of percentages in 1,000 pounds per acre of complete fertilizer.

The complete fertilizer (NPK) has a ratio $N:P_2O_5:K_2O=6:10:8$. Applications (per plot, one hundredth acre) to this plot were at the rate of 4 pounds of $NaNO_3$, 6.25 pounds of superphosphate, and 1.666 pounds of KCl, and this treatment is hereafter referred to as the standard (S) application (*3*).

METHODS OF SAMPLING

Details of the method of sampling have been given in an earlier publication (*6*).

Leaves from stems of plants that were abnormal in relation to the average of the particular experimental plot were not sampled. The first samples were taken as soon as the plants were sufficiently developed to permit the ensemble of leaves in a row to supply the amount

of material sufficient for analysis. The other samples were collected at intervals.

The leaves were dried in a drying oven immediately after being brought from the field and were then ground in a Wiley mill.

PRESENTATION AND DISCUSSION OF RESULTS

It will simplify the interpretation of results to divide the plots according to treatments, as follows:

(1) Plots receiving the standard fertilizer (S) and those with one or two of the elements omitted, viz, plots receiving N, P, K, NP, NK, PK, NPK.

(2) Plots receiving different proportions of N, P, and K in complete fertilizers, i. e., plots receiving complete fertilizers differently equilibrated—viz, plots 0.5 (NPK), 2(NPK), (1.5N)PK, N(1.5P)K, NP(1.5K), and also 30 tons of manure.

APPEARANCE OF THE LEAVES

The appearance of the leaves of plants receiving different treatments was markedly different.

The N plants, which were small and erect, had small leaves, rugose or wavy along the margins, and dark green; many were folded upward along the midrib.

The P plants were a little larger and less erect than the N plants; the leaves were larger and a little lighter in color, but otherwise not very different from those on the N plots.

The K plants were about the same size as those on the N plots, but the leaves were distinctly larger, smoother, and lighter in color.

The NP plants were somewhat larger and darker green that those on the P plots, but similar in other respects.

The NK plants were about the same size as those on the P plots, but the leaves were smoother; they were a little lighter in color than those on the NP plots.

The PK plants were distinctly larger and more spreading than any of the foregoing, and the leaves were fairly large, nearly smooth, and lighter green than those on the other plots, except possibly on the manure plots.

The NPK plants were a little smaller and less spreading than those on the PK plots; the leaves were a little smoother and lighter in color and also little larger than those on the NP plots.

The "manure" plants were the largest of all, and were less spreading and slightly darker green than those on the PK plots; the leaves were folded somewhat, but were little waved along the margins.

The (1.5N) PK plants were about the same in most respects as those on the NPK plots, though they appeared a little less vigorous and slightly darker green.

The N (1.5P) K plants were larger than those on the NPK plots, and the leaves were larger and less folded; the color of the leaves was about the same as that on the NPK plots.

The NP (1.5K) plants were more spreading than those the NPK plots, and the leaves were smoother, larger, and lighter green.

The 0.5 (NPK) plants closely resembled the N plants, but they were a little lighter in color.

The 2 (NPK) plants resembled those on the NP (1.5K) plots, but they were a little larger and darker green, and the leaves were more folded.

DEVELOPMENT OF THE PLANTS

Method of Determining Development

The development of the plants was determined in the following manner:

Plants were taken from the two halves of guard strips adjacent to a given plot, which received the same fertilizer treatment as the plots being sampled. At each date of sampling for plant development, which was within a day or two after the date of leaf sampling for foliar diagnosis, a plant was dug from every third row lengthwise of the plot, and intervening rows were sampled at later dates. Eight plants, half from each guard strip, constituted a sample on each date.

There is a possibility that some of the roots of these plants extended beyond the area receiving the particular fertilizer treatment into the area receiving the treatment of the adjacent plot. If this occurred, the effect was not sufficient to appear in the tops of the plants, which were apparently like those of the plot which they were considered to represent. This method was followed to avoid disturbing plants on the plots proper, the yields of which would very likely have been influenced by the removal of plants, even if corrections had been made for the missing plants.

The roots of the plants were brushed free from adhering soil, and the individual plants were weighed immediately, first entirely and then with tops removed. The data for all underground portions, including roots and tubers, were recorded together. At the first sampling, roots constituted most of the combined weight, but at later samplings the tubers accounted for an increasingly greater part of the weight. Original seed pieces which in many cases were attached to the plants were removed before weighing was done.

The plants developed in a regularly progressive manner without any injurious factors affecting growth. The development of the aerial parts of the plants from duplicate plots varied greatly in some plots, and especially in the following pairs: NPK (Nos. 4–8 and 1–10); PK (Nos. 1–8 and 4–10); K (Nos. 1–4 and 4–14); and (1.5N) PK (Nos. 2–4 and 5–14). The development was more nearly alike in plants from duplicate plots that did not receive potash; that is, in N (Nos. 1–2 and 4–16), NP (Nos. 1–6 and 4–12), and P (Nos. 1–3 and 4–15). This fact suggests that potash nutrition is a dominant factor in determining the great differences in the development of the plants in some of the duplicate plots; it may be related to the differences in the supply of water (*4*) or to other factors associated with soil heterogeneity.

Relation of Rate of Growth During the Early Part of the Growth Period to Final Yield of Tubers

Table 2 gives the weights of the aerial parts and of the tubers on four different dates—July 4, July 25, August 10, and August 24. Table 3 gives the aerial development as an indication of the growth rate during the period from July 4 to July 25, together with the final yield of tubers.

The parallelism within the plots of a tier is within a sufficient approximation for a field experiment. This correlation may not always be found, although it has long been known from field observations that fertilizers have a marked influence on the rate of growth of plants during the early part of their vegetative development. Barring in-

jurious factors later in the season, the general appearance of crops during the early stages has been recognized by agronomists as affording a good basis for the prediction of yields. The impulse received at the beginning of vegetative growth is characteristic of the mineral nutrition during the whole course of development and the yield often confirms this fact. Hence, for comparative studies, more confidence may be placed in the data for the rapidity of growth as an indication of the response to treatment than in the final yields themselves.

TABLE 2.—*Development of tops and of tubers, from potato plants receiving the standard and the incomplete fertilizers*

[Fresh weights per plant]

Treatment	Tier No.	Plot No.	Portion	July 4	July 25	Aug. 10	Aug. 24
				Grams	*Grams*	*Grams*	*Grams*
N	1	2	Aerial	59.9	162.5	311.3	311.3
			Tubers	17.2	61.3	173.7	273.7
	4	16	Aerial	42.0	151.3	232.5	301.3
			Tubers	14.6	41.2	167.5	275.0
NPK	1	10	Aerial	111.1	425.0	402.5	357.5
			Tubers	20.9	175.0	272.5	466.3
	4	8	Aerial	140.8	500.0	517.5	532.5
			Tubers	25.3	170.0	330.0	565.0
PK	1	8	Aerial	108.0	360.0	327.1	320.0
			Tubers	25.1	212.5	310.0	486.3
	4	10	Aerial	89.0	225.7	407.1	425.0
			Tubers	17.4	122.9	331.5	547.5
NK	1	7	Aerial	85.5	325.0	451.3	386.3
			Tubers	23.4	97.5	275.0	481.2
	4	11	Aerial	53.5	355.0	501.3	380.0
			Tubers	12.4	117.5	278.7	336.3
NP	1	6	Aerial	93.8	306.3	273.8	386.3
			Tubers	21.7	112.5	216.2	457.5
	4	12	Aerial	78.4	307.1	240.0	388.8
			Tubers	18.0	92.9	160.0	352.5
K	1	4	Aerial	60.8	220.0	281.3	356.3
			Tubers	17.3	74.3	213.7	481.2
	4	14	Aerial	31.1	94.3	165.7	170.0
			Tubers	13.0	65.7	140.0	201.3
P	1	3	Aerial	75.5	196.3	246.3	200.0
			Tubers	18.5	86.2	210.0	302.5
	4	15	Aerial	53.8	157.5	153.8	125.0
			Tubers	17.8	108.8	168.7	227.5

TABLE 3.—*Relation between rate of growth and final yields from plants receiving the standard and the incomplete fertilizers*

Treatment	Tier No.	Plot No.	Weight addition to aerial portions, per plant, between July 4 and July 25	Class order	Final yield per plot	Class order
			Grams		*Pounds*	
N	1	2	112.6	7	109	7
	4	16	109.3	5	88	5
NPK	1	10	313.9	1	162	2
	4	8	359.2	1	195	2
PK	1	8	252.0	2	148	4
	4	10	136.7	4	208	1
NK	1	7	239.5	3	163	1
	4	11	301.5	2	174	3
NP	1	6	212.5	4	124	5
	4	12	228.7	3	140	4
K	1	4	169.2	5	155	3
	4	14	63.2	7	70	7
P	1	3	120.8	6	114	6
	4	15	103.7	6	88	6

In this case, the coefficient of correlation between the final yield and the growth of the aerial parts between July 4 and July 25, computed by the usual method, is $+0.785 \pm 0.0692$, which may be considered highly significant.

The presentation of another magnitude—in this case growth rate—in addition to that represented by the final yields, is, from an agronomical standpoint, logically superior to the practice of blindly increasing the number of observations of yields from which to calculate the probable error.

COMPOSITION OF LEAVES FROM PLANTS RECEIVING THE COMPLETE STANDARD FERTILIZER AND THOSE WITH EITHER ONE OR TWO OF THE ELEMENTS OMITTED

The results of the analyses for N, P_2O_5, and K_2O on four sampling dates, expressed as percentage of the dry weight of material, are given in table 4 and are represented graphically in figure 1.

TABLE 4.—*Nitrogen, phosphoric acid, and potash content on different dates of the fourth leaf of potato plants receiving the standard and the incomplete fertilizers; expressed on a dry-weight basis*

Treatment	Tier No.	Plot No.	Mineral nutrient	July 7	July 29	Aug. 9	Aug. 24
				Percent	*Percent*	*Percent*	*Percent*
N	1	2	N	5.12	4.42	4.15	3.64
			P_2O_5	.404	.534	.472	.344
			K_2O	3.97	2.54	2.01	1.77
	4	16	N	4.71	4.11	3.75	3.40
			P_2O_5	.424	.502	.458	.362
			K_2O	4.438	3.139	2.635	2.151
NPK	1	10	N	4.98	3.88	3.62	3.14
			P_2O_5	.584	.509	.482	.392
			K_2O	6.589	4.337	4.697	4.348
	4	8	N	4.92	4.36	3.62	3.23
			P_2O_5	.604	.542	.460	.398
			K_2O	6.907	5.129	5.000	4.961
PK	1	8	N	4.55	3.36	3.08	2.82
			P_2O_5	.624	.510	.496	.430
			K_2O	6.759	5.321	5.348	5.038
	4	10	N	4.45	3.64	3.10	2.75
			P_2O_5	.636	.580	.484	.370
			K_2O	6.651	5.193	5.062	5.300
NK	1	7	N	5.10	4.03	3.78	3.26
			P_2O_5	.464	.510	.452	.352
			K_2O	6.628	5.554	5.279	5.457
	4	11	N	5.04	4.31	3.78	3.36
			P_2O_5	.412	.498	.420	.332
			K_2O	6.790	5.968	6.269	6.434
NP	1	6	N	5.14	3.87	3.71	3.36
			P_2O_5	.590	.532	.486	.396
			K_2O	2.531	1.519	1.186	1.085
	4	12	N	5.08	4.20	3.78	3.37
			P_2O_5	.558	.526	.480	.412
			K_2O	4.632	2.635	2.170	1.755
K	1	4	N	4.61	3.87	3.39	2.78
			P_2O_5	.424	.540	.464	.370
			K_2O	6.318	5.217	4.915	5.000
	4	14	N	4.10	3.50	3.20	2.56
			P_2O_5	.432	.452	.432	.328
			K_2O	6.317	5.232	5.496	5.242
P	1	3	N	4.57	3.46	3.24	2.79
			P_2O_5	.592	.521	.520	.450
			K_2O	2.41	1.64	1.29	1.11
	4	15	N	4.30	3.38	3.06	2.34
			P_2O_5	.548	.512	.514	.444
			K_2O	5.15	3.488	3.04	2.35

GENERAL CHARACTERISTICS OF THE GRAPHS OF PLOTS RECEIVING THE COMPLETE STANDARD FERTILIZER AND THOSE WITH EITHER ONE OR TWO OF THE ELEMENTS OMITTED

The differences between the foliar diagnosis of plants from plots receiving different treatments are much greater than between duplicate pairs receiving the same treatment (fig. 1). On soils much more

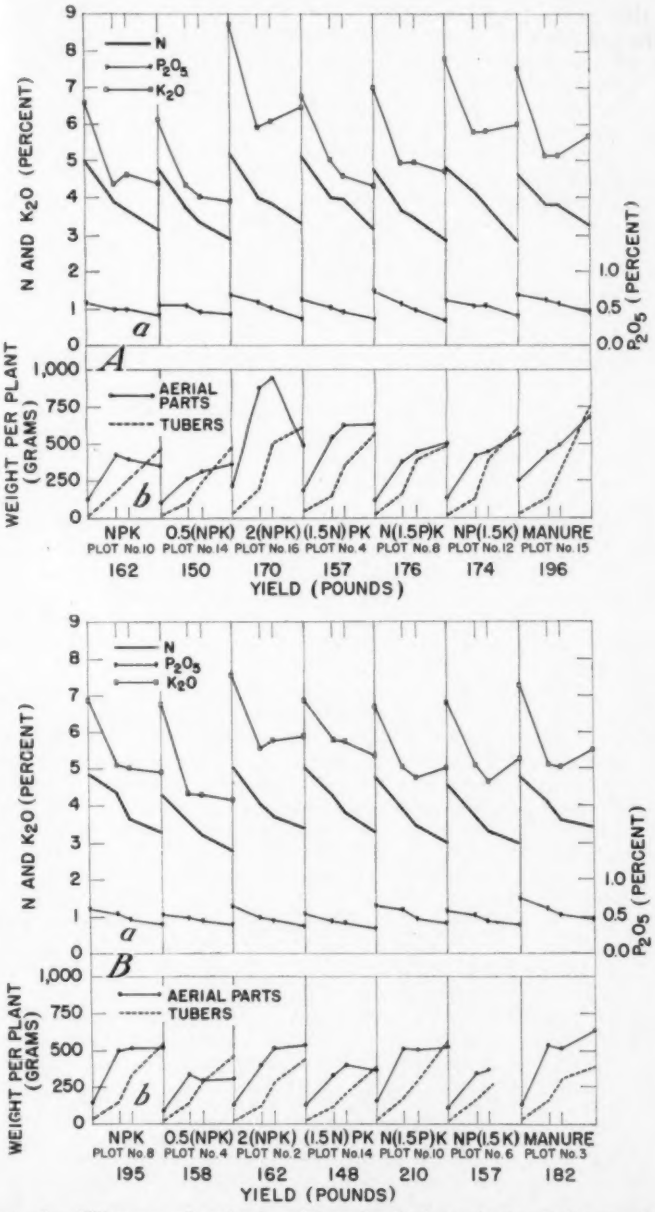

FIGURE 1.—Nitrogen, phosphoric acid, and potash contents of the fourth leaf (dry foliage) of potato plants at four periods of sampling (*a*), and the weights of aerial parts and of tubers at corresponding dates (*b*) from plots receiving the standard and the incomplete fertilizers: *A*, tier 1; *B*, tier 4.

uniform than the soil of these experiments, it has, as already stated, been established that the foliar diagnoses of plants growing on plots receiving the same treatment, the yields of which are as nearly identical as can be expected under field conditions, coincide.

In the present experiments the foliar diagnoses for nitrogen and phosphoric acid of plants on similarly treated plots are, in general, concordant, within a sufficient approximation for field experiments (as compared with the differences existing between the foliar diagnosis of plants receiving different treatments). The divergences of the graphs for potash in many duplicate plots, however, are very great. This is the result of differences in topography, the exposure of the subsurface clay horizons being greater in some plots than in others. This was accompanied by great differences in the potash nutrition of the plants between certain duplicate plots, especially in the NPK, NP, NK, K, and P plots.

A preliminary examination of the results shows the following characteristics:

Nitrogen.—In all plots of tiers 1 receiving nitrogen addition, the content in terms of the dry weight of the leaf is 5.00 percent (4.71 in tier 4) or more on July 7, the first sampling date, and in all plots which did not receive nitrogen fertilizer applications it is 4.61 (4.45 in tier 4) or less. An increase in the content of N is reflected in all successive samplings in the leaves of plants which received nitrogen additions over those which did not. The nitrogen decreases with the age of the leaf in all cases.

Phosphoric acid.—The phosphoric acid content of leaves from plots in tier 1 treated with superphosphate is 0.584 percent (0.548 in tier 4) or more on July 7, as compared with 0.464 percent (0.424 in tier 4) or less on plots not receiving this fertilizer.

The characteristic of the phosphoric acid graphs in all tiers is the attainment of a maximum on July 29 (second sampling date) on all plots which did not receive phosphate applications. This maximum is not present in the leaves of any plot which received phosphate additions; it appears to be a temperature effect and may be masked in plots which received phosphate additions because of more rapid assimilation. The nitrogen graphs confirm this view, for whenever there is a rapid assimilation of nitrogen, as in the plots which received phosphate, a maximum in the phosphoric acid graphs is not present after the first sampling date.

Potash.—The leaves of plants from all plots which received potash additions contain 6.318 percent (6.317 in tier 4) K_2O or more on July 7, as compared with 3.97 percent (5.15 in tier 4) or less in plots which received no potash fertilizer. These relationships are maintained throughout successive samplings. The potash decreases regularly with the age of the leaf except that when insufficient nitrogen or phosphoric acid is present the latter accumulates in the leaf during the later stages of growth.

The effect factors of each element (N or P or K) are related to the presence of the others in such a way that:

(1) The omission of nitrogen from the complete fertilizer (S) has resulted (compare NPK and PK) in an increase in both the potassium and phosphorus content of the leaf in tier 1 throughout the whole period, and in tier 4 in an increase of the phosphorus through the first three periods, and of the potassium in the last three periods.

The omission of nitrogen from the complete fertilizer is accompanied by a retardation in the development of the aerial parts in both tiers 1 and 4.

(2) The omission of phosphorus from the standard complete fertilizer (S) is accompanied (compare NPK and NK) by an increase in the nitrogen and potassium content, but, as the slopes of the graphs indicate, with smaller utilization of both elements.

(3) The omission of potassium from the complete fertilizer (S) is accompanied (compare NPK with NP) by a slight increase in the nitrogen and phosphorus content of the leaf throughout the period. Associated with this is a generally slower development of the aerial parts and decrease in yeild of tubers. The omission of potassium has resulted in a much lower content of potassium in plot 1–6 than in its duplicate, 4–12, resulting in much lower yields.

Reasons for Comparing the Foliar Diagnosis of Plants on Each Plot With That of Plants on the N Plot

When the experimental plots were laid out in 1916 each tier contained five unfertilized check plots, viz, plot 1 and each fourth plot following it. In 1923, however, and in succeeding years until 1932, the check plots were fertilized uniformly with 2.67 pounds of sodium nitrate and 6.25 pounds of 16 percent superphosphate. A comparison of the yields for the two periods 1918–20 and 1921–22 (*3*) shows that the N plots, which were superior at first (1918–20) to the check plots, later became inferior to them (1921–22). The foliar diagnosis of the plants on the N plots supplies the explanation. The progressive addition of nitrogen has thrown the relationships of N, P_2O_5, and K_2O out of balance, by reducing the absorption of P and K.

In the following analysis of data of tier 1, therefore, all of the plots from No. 3 to No. 10 will be compared with plot No. 2 of this tier, viz, the N plot, since this is the lowest yielding plot. Before doing so, the N plot (1–2) will be discussed in detail to illustrate the characteristic properties of the graphs (foliar diagnosis). It will be shown that the differences in foliar diagnosis between the differentially treated plots correspond to important differences in the mode of vegetative growth and of the development of tubers.

Foliar Diagnosis of Plants on the N Plot as Illustrative of the Method of Interpretation

The graphs for the N plot represent the foliar diagnosis, as defined (p. 397), for N, P_2O_5, and K_2O of the Rural Russet variety for the year 1935. The foliar diagnosis represents an analytical characteristic of the chemism or chemical state of the plants on the N plot (No. 2) of tier 1 at the moment of sampling the leaves. This chemical condition is based on the dry matter of the leaf without taking into consideration the weight of the dry material at each sampling or the number of leaves sampled from each plant.

It is now clear from the definitions that no physiological significance whatever can be attributed to the graphs of the N plot when considered alone, but only in its relation to other plots. Hence, the graphs considered independently of all other field data and all other foliar diagnoses have no physiological or agronomical significance.

Certain inferences, however, may be drawn from the graphs of the N plot (1–2). For example, the foliar diagnosis of the plants on this

plot shows the lowest phosphoric acid content and the poorest assimilation of this element of any of the plots, and is accompanied by slow development of the plants; small, wrinkled, dark-green leaves; and low yield. Furthermore, these characteristics are reproduced in the duplicate N plot (4–16).

COMPARISON OF THE GRAPHS OF THE DIFFERENTLY TREATED PLOTS WITH THE CONTROL (N-TREATED) PLOT[5]

The following comparisons of foliar diagnoses and plant development in different treatments with that in N are based on graphs shown in figure 1 and on data presented in tables 2 and 4. The foliar diagnoses of plants on the plots in tier 1 are in general, and with a sufficient approximation for field experiments, reproduced in the respective duplicate plot in tier 4. Such divergences as exist are the result of soil heterogeneity. The same comments refer also to the foliar diagnosis of the plants of the plots in tier 2 as compared with their duplicates in tier 5.

Comparison of the NPK Plot (1–10) With the N Plot (1–2)

Nitrogen of NPK is lower than that of N throughout the period. The relative steepness of the slopes of the graphs (relative decrease with time) is greater in NPK during the early period (a decline of 1.10 percent for NPK as compared with 0.70 percent for N), indicating a greater evacuation of nitrogen from the leaves of plants growing on the complete fertilizer plot as compared with those on the plot receiving nitrogen alone. This relative excess of export over import is an indication of greater demand by the plant.

The phosphoric acid graphs have neither the same position nor the same form. The graph of NPK is much higher than that of N on July 7—0.58 percent for NPK, as compared with 0.40 percent for N—and, whereas phosphoric acid continues to decline with time in NPK throughout the period, a maximum is shown on July 29 in N. Reference has already been made to this maximum in the plots which did not receive phosphate additions. The decrease from July 7 to August 24 in N is only 0.06 percent, as compared with 0.19 percent in NPK.

The potash graphs also are very unlike in form and position in these plots. Potash is much higher in the leaves of plants growing on the complete fertilizer plot throughout the whole period (the range is from 6.59 to 4.35 percent for NPK and from 3.97 to 1.77 percent for N). In both plots the slope is very steep during the first period, but in NPK a reversal of direction takes place, resulting in a maximum on August 9—an indication that the leaves of the plants growing on the NPK plot have received more potash than has been assimilated by the plant during the second period.

Related to these differences is a much more rapid and luxuriant development of the aerial parts in NPK, in which complete development of the tops has occurred by the end of July. The yield of tubers of NPK is 40 percent greater than in N. These differences are qualitatively reproduced in tier 4.

[5] For the sake of brevity in the discussion that follows, the plot-treatment symbol is used to refer to leaves from plants growing on the plot that received the treatment indicated by the symbol.

Comparison of the PK Plot (1–8) With the N Plot (1–2)

The nitrogen in the leaves of PK is much lower throughout the period than in N. The range is from 4.55 to 2.82 percent for PK and from 5.12 to 3.64 percent for N. The form of the nitrogen graphs of these two plots also is unlike.

It may be noted here that the nitrogen graph of PK is the lowest of all on July 7 and the range corresponds closely with that of K and P.

Phosphoric acid in PK is higher throughout the period and its graph is similar in form to that of phosphoric acid in NPK. No maximum occurs in PK on July 29 as it does in N.

Potash is very much higher in PK than in N. The range is from 6.76 to 5.04 percent for PK and from 3.97 to 1.77 percent for N. At the first sampling the relative values are as wide apart as 6.76 percent and 3.97 percent. The slope of the graph of PK begins to decrease during the second and third periods, showing that evacuation from the leaf is less rapid than in N.

Related to these different foliar diagnoses is a more rapid development of the aerial portion of PK as compared with that of N, especially during the early period. The relative weights of tops per plant in PK and in N on July 25 are 360 g and 162.5 g, respectively. On August 24 the relative yields per plant are 486 g and 274 g, respectively.

It is of interest to note that up to August 24 the yield of tubers per plant for PK exceeds that of NPK, this superiority does not continue as the final yields from NPK are much greater than those from PK.

These differences are qualitatively reproduced in tier 4.

Comparison of the NK Plot (1–7) With the N Plot (1–2)

Nitrogen is somewhat lower and the slope is steeper in NK during the period July 7 to August 24. The range is from 5.10 to 3.26 percent for NK and from 5.12 to 3.64 percent for N.

The phosphoric acid graphs resemble one another in form, but the graph of NK is higher at the first and last sampling dates than that of N. The range is from 0.464 to 0.352 percent for the former and from 0.404 to 0.344 percent for the latter. The maximum already spoken of on July 29 appears in both, although it is higher in N.

Potash is very much higher in NK. The relative ranges are from 6.63 to 5.46 percent for NK and from 3.97 to 1.77 percent for N. The form of the graphs also is very different. Thus, the graph of NK does not descend regularly with time. After the second period the graph of NK reverses direction, indicating that the leaves are receiving more potash than is being assimilated by the plant.

Related to these differences in the foliar diagnosis, the development of the aerial parts and the yield of tubers are much greater in NK than in N. The relative weights per plant of the aerial parts on July 4 are 85.5 g for NK and 59.9 g for N, and on August 24, 386.3 g and 311.3 g, respectively. The yield of tubers on August 24 is 481.2 g for NK and 273.7 g for N.

Comparison of the NP Plot (1–6) With the N Plot (1–2)

Except for an insignificant difference at the first sampling date, nitrogen is lower in NP than in N. The range is from 5.14 to 3.36 percent for NP and from 5.12 to 3.64 percent for N. The relatively greater slope of the graph of NP, especially during the period from

July 7 to July 29, indicates greater utilization of N and at a high level.

Phosphoric acid is nearly 50 percent greater at the first sampling date in NP than in N. The range is from 0.59 to 0.40 percent for NP and only from 0.40 to 0.34 percent for N. A maximum does not occur on July 29 in NP as in N.

Potash is much lower in NP. The range is from 2.53 to 1.08 percent for NP and from 3.97 to 1.77 percent for N. The addition of phosphate, then, has resulted in a decreased absorption of potassium.

The differences shown in the foliar diagnosis have resulted in improved development of the aerial parts and of tubers of NP over those of N. On July 24 the relative weights of tops per plant are 306 g for NP as compared with 162 g for N; and of tubers on August 24, 445 g, as compared with 273 g. The development and yield of the plants growing on plot 1–6, however, are below those of NPK (1–10, 4–8), NK (1–7, 4–11), and PK (1–8, 4–10).

Comparison of the K Plot (1–4) With the N Plot (1–2)

Nitrogen is lower throughout the season in K than in N. The range is from 4.61 to 2.78 percent for K and from 5.12 to 3.64 percent for N, and the slope is greater in K than in N. There is, therefore, more demand on the nitrogen of plants growing on the K plot, which is related to a low level of supply of this element.

Phosphoric acid is somewhat higher in K than in N, although the graphs of both plots are similar in form. The ranges are from 0.424 to 0.370 percent and 0.404 to 0.344 percent in K and N, respectively. Both have a maximum at the second sampling date on July 29. The graphs nearly coincide after the second period.

Potash is very much higher in K than in N. The range is from 6.32 to 5.00 percent in K and from 3.97 to 1.77 percent in N. The a cent of the graph of K during the third period (July 29–August 9) indicates an accumulation of potassium. This phenomenon was noted also in NK (in NPK accumulation occurs during the second period).

Related to these differences in the foliar diagnosis is a superior development of the plants growing on the K plot. The relative weights of tops per plant on July 24 are 230 g for K and 162 g for N, and of tubers on August 24, 481 g and 274 g, respectively.

Although the development of the aerial parts of the plants growing on the K plot is, as in the N plot, relatively slow at first, there is no decrease in the weight of tops as the plant ages as is the case in NPK, PK, NK, NP, and P. The weight of the tubers of PK increases rapidly after July 29. On August 10 the weight of tubers surpasses even that of NPK, but the superiority does not continue.

Comparison of the P Plot (1–3) With the N Plot (1–2)

Nitrogen is much lower throughout the whole period in P than in N. The range is from 4.57 to 2.79 percent for P and from 5.12 to 3.64 percent for N. The forms of the graphs are similar except that the gradient is steeper in P from July 7 to July 29. The nitrogen graph of P coincides very nearly with that of K and with PK.

Phosphoric acid is higher (except on July 29) throughout the period in P. The ranges are from 0.59 to 0.45 percent and from 0.40 to 0.34 percent for P and N, respectively. There is no maximum on July 29 in P.

Potash is lower throughout the whole period in P than in N. The range is from 2.41 to 1.11 percent for P and from 3.97 to 1.77 percent for N.

Related to these differences in the foliar diagnosis is a slightly improved growth of tops of plants growing on the P plots during the early period, after which both nitrogen and potassium become limiting factors. On July 29 the relative weights of the aerial parts per plant are 196 g for P and 162 g for N, and the yields of tubers on August 24 are 302 g and 273 g for P and N, respectively. The P plot shows the poorest development of tops of any of the plots after July 29, but the yield of tubers, as already indicated, is higher than in the N plot.

RELATION OF DEVELOPMENT TO FOLIAR DIAGNOSIS OF PLANTS RECEIVING DIFFERENT PROPORTIONS OF N, P_2O_5, AND K_2O IN COMPLETE FERTILIZERS AND THOSE RECEIVING THE STANDARD APPLICATION

The development of plants and the composition of the fourth leaf at the different sampling dates in plots receiving different proportions of N, P_2O_5, and K_2O in complete fertilizers are shown in tables 5 and 6, and in figure 2.

TABLE 5.—*Development of tops and of tubers from potato plants receiving different proportions of nitrogen, phosphoric acid, and potash in complete fertilizers*

[Fresh weights per plant]

Treatment	Tier No.	Plot No.	Portion	July 4	July 25	August 10	August 24
				Grams	*Grams*	*Grams*	*Grams*
0.5(NPK)	2	14	Aerial	87.9	267.5	311.3	372.5
			Tubers	19.6	110.0	256.2	487.5
	5	4	Aerial	89.4	345.0	296.3	311.3
			Tubers	21.6	160.0	290.0	462.5
2(NPK)	2	16	Aerial	200.3	872.5	945.0	492.5
			Tubers	24.5	295.0	492.5	611.0
	5	2	Aerial	120.6	407.5	515.0	540.0
			Tubers	22.4	132.5	281.3	450.0
(1.5 N)PK	2	4	Aerial	183.3	543.8	635.0	648.8
			Tubers	29.2	157.5	355.0	583.7
	5	14	Aerial	114.4	341.3	407.5	375.0
			Tubers	23.5	113.7	212.5	381.3
N(1.5 P)K	2	8	Aerial	119.6	387.5	448.8	502.9
			Tubers	21.8	163.8	392.5	494.2
	5	10	Aerial	140.8	520.0	517.5	532.5
			Tubers	25.3	190.0	330.0	565.0
NP(1.5 K)	2	12	Aerial	124.9	425.7	457.5	582.5
			Tubers	19.7	137.2	392.5	621.3
	5	6	Aerial	103.0	348.8	370.0	----------
			Tubers	20.0	176.2	256.3	----------
Manure	1	15	Aerial	251.9	446.3	591.3	692.5
			Tubers	31.1	152.5	308.7	753.8
	4	3	Aerial	125.4	545.0	525.0	635.0
			Tubers	22.1	173.0	313.8	392.5

COMPARISON OF THE 0.5 (NPK) PLOT (2–14) WITH THE NPK PLOT (1–10)

Nitrogen is lower throughout the period in 0.5 (NPK) than in NPK. The range is from 4.84 to 2.84 percent for 0.5 (NPK) and from 4.98 to 3.14 percent for NPK.

Phosphoric acid also is lower in 0.5 (NPK) except at the second and the last periods. The range is from 0.52 to 0.42 percent for 0.5 (NPK) and from 0.58 to 0.39 percent for NPK.

TABLE 6.—*Nitrogen, phosphoric acid, and potash content on different dates of the fourth leaf of potato plants receiving different proportions of these minerals in complete fertilizers, expressed on a dry-weather basis*

Treatment	Tier No.	Plot No.	Mineral nutrient	July 7	July 29	August 9	August 24
				Percent	*Percent*	*Percent*	*Percent*
0.5 (NPK)	2	14	N	4.84	3.77	3.34	2.84
			P_2O_5	.520	.528	.466	.418
			K_2O	6.108	4.379	4.031	3.920
	5	4	N	4.34	3.57	3.12	2.75
			P_2O_5	.524	.486	.454	.400
			K_2O	6.705	4.379	4.310	4.162
2 (NPK)	2	16	N	5.23	4.04	3.87	3.30
			P_2O_5	.682	.562	.500	.354
			K_2O	8.771	5.920	6.124	6.453
	5	2	N	5.03	4.08	3.76	3.35
			P_2O_5	.650	.509	.454	.368
			K_2O	7.521	5.50	5.658	5.775
(1.5 N) PK	2	4	N	5.23	4.00	3.98	3.16
			P_2O_5	.614	.488	.464	.356
			K_2O	6.780	5.040	4.534	4.302
	5	14	N	4.98	4.26	3.76	3.23
			P_2O_5	.528	.446	.430	.348
			K_2O	6.814	5.744	5.704	5.310
N (1.5 P) K	2	8	N	4.84	3.72	3.46	2.86
			P_2O_5	.710	.564	.482	.350
			K_2O	6.969	4.914	4.934	4.670
	5	10	N	4.84	3.90	3.40	2.96
			P_2O_5	.658	.608	.576	.404
			K_2O	6.678	5.031	4.883	4.961
NP (1.5 K)	2	12	N	4.86	4.19	3.71	2.82
			P_2O_5	.594	.522	.513	.394
			K_2O	7.798	5.767	5.782	5.996
	5	6	N	4.54	3.71	3.26	3.01
			P_2O_5	.554	.502	.444	.382
			K_2O	6.794	5.124	4.653	5.310
Manure	1	15	N	4.65	3.80	3.79	3.28
			P_2O_5	.688	.608	.564	.448
			K_2O	7.550	5.128	5.155	4.658
	4	3	N	4.84	4.04	3.58	3.45
			P_2O_5	.750	.604	.502	.472
			K_2O	7.248	5.116	4.988	5.601

Potash is lower except in the second period in 0.5 (NPK). The range is from 6.11 to 3.92 percent for 0.5 (NPK) and from 6.59 to 4.35 percent for NPK. The forms of the graphs are different. No accumulation of potassium occurs after the second period in 0.5 (NPK) as in NPK.

Corresponding to these differences in the foliar diagnosis is a much slower development of the aerial parts of 0.5 (NPK) during the early part of the season. The yield of tubers of 0.5 (NPK) is 8 percent below that of NPK.

COMPARISON OF THE 2 (NPK) PLOT (2–16) WITH THE NPK PLOT (1–10)

Nitrogen is higher throughout in 2 (NPK). The range is from 5.23 to 3.30 percent for 2 (NPK) and from 4.98 to 3.14 percent for NPK. The forms of the graphs have the same characteristics in both plots.

Phosphoric acid also is higher in 2 (NPK) except during the last period of growth. The range is from 0.68 to 0.35 percent for 2 (NPK) and from 0.58 to 0.39 percent for NPK. The relative steepness of the slope of the 2 (NPK) graph shows that greater evacuation of phosphorus has taken place, indicating better assimilation and at a high level.

Potash is very much higher throughout the whole period in 2 (NPK). The range is from 8.77 to 6.45 percent for 2 (NPK) and from 6.59 to 4.34 percent for NPK.

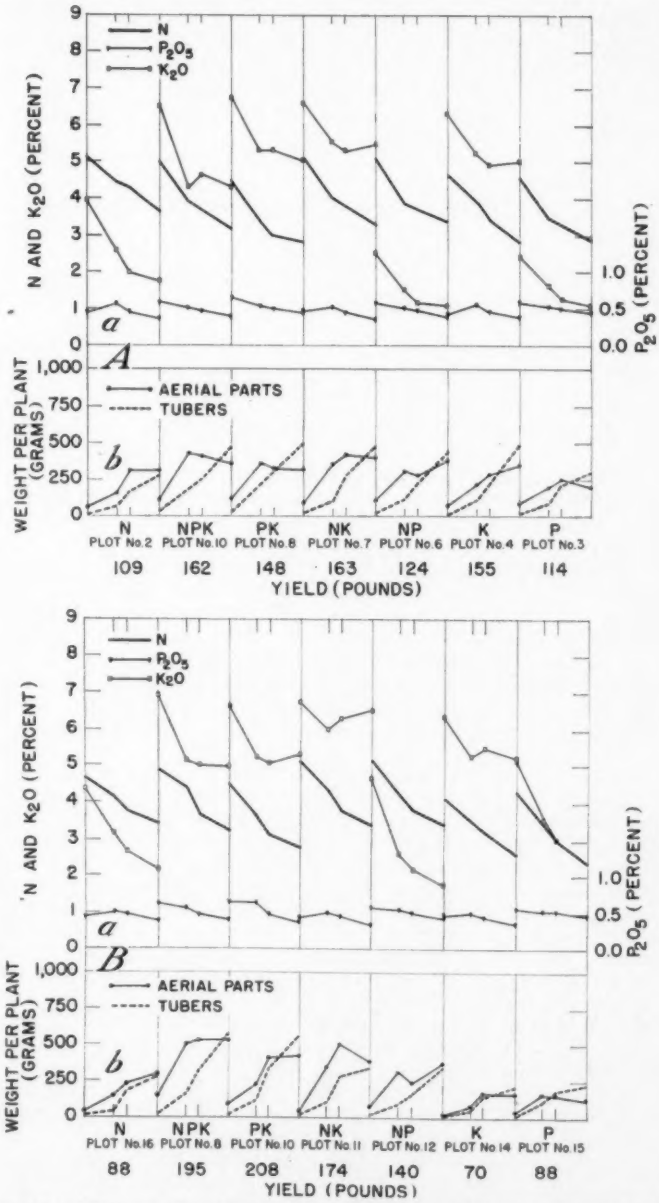

FIGURE 2.—Nitrogen, phosphoric acid, and potash content of the fourth leaf (dry foliage) of potato plants at four periods of sampling (*a*), and the weights of aerial parts and of tubers at corresponding dates (*b*) from plots receiving different proportions of these minerals in complete fertilizers: *A*, Tiers 1 and 2; *B*, tiers 4 and 5.

The potash content of the leaves of 2 (NPK) is higher than that of leaves from any other plot. But it cannot at the present stage be determined whether this high potassium content in the leaves of plants from plot 2–16 has resulted in any injury to development or yield. It is possible that the rapid loss of weight of tops referred to in the next paragraph is due to this cause. The fact that this loss does not occur in 5–2 where the potassium content is lower lends confirmation to this view. But what would be the limit of potassium absorption above which injury would be shown and where the boundary of cessation of buffer action lies are not disclosed by this experiment.

After July 29 both graphs reverse direction, but whereas that of 2 (NPK) continues its ascent that of NPK declines. It is significant that this accumulation of potassium occurs among the relatively high yielding plots only.

Related to these differences in the foliar diagnosis is a very luxuriant growth of tops in 2 (NPK), followed by a rapid loss in weight after August 10. The yield of tubers has been increased 28 percent.

Comparison of the (1.5N) PK Plot (2–4) With the NPK Plot (1–10)

Nitrogen is a little higher in (1.5N) PK than in NPK. The range is from 5.23 to 3.16 for the former and 4.98 to 3.14 percent for the latter. The forms of the graphs, however, are much alike.

Phosphoric acid is slightly higher in (1.5N) PK. The range is from 0.61 to 0.36 percent for (1.5N) PK and from 0.58 to 0.39 percent for NPK. The forms of the graphs are somewhat similar.

Potash is somewhat higher throughout the first two periods in (1.5N) PK than in NPK. The forms of the graphs are quite different.

Related to these differences in the foliar diagnosis, the vegetative growth of plants on the (1.5N) PK plot far surpasses that of plants on the NPK plot throughout the period, and the weights of tubers of the former on August 24 are greater than those of the latter.

Comparison of the N (1.5P) K Plot (2–8) With the NPK Plot (1–10)

Nitrogen is slightly lower throughout the period in N (1.5P) K. The range is from 4.84 to 2.86 percent for this plot and from 4.98 to 3.14 percent for NPK. The forms of the graphs, however, are similar.

Phosphoric acid is very much higher in N (1.5P) K during the first two periods, after which greater utilization in N (1.5P) K causes the graft to decend below that of NPK. The range is from 0.71 to 0.35 percent for this treatment and from 0.58 to 0.39 percent for NPK. The relative steepness of the slope of N (1.5P) K indicates a better utilization of phosphorus than in NPK.

Potash is higher throughout the period in N (1.5P) K. The range is from 6.97 to 4.67 percent, as compared with 6.59 to 4.35 percent for NPK. The forms of the graphs are somewhat similar, however.

Related to these differences in the foliar diagnosis is the fact that, although from July 4 to July 25 the vegetative growth and development of tubers are nearly identical in N (1.5P) K and NPK, vegetative growth continues in the plants growing on the former plot whereas a loss of weight is shown in those on the latter. The yield of tubers is 8 percent greater in N (1.5P) K than in NPK.

Comparison of the NP(1.5K) Plot (2–12) With the NPK Plot (1–10)

Nitrogen is a little lower during the first and fourth periods in NP(1.5K), ranging from 4.86 to 2.82 percent, as compared with 4.98 to 3.14 percent for NPK, but the forms of the graphs are similar.

The phosphoric acid graphs almost coincide; they range from 0.59 to 0.39 percent and from 0.58 to 0.39 percent for NP(1.5K) and NPK, respectively.

Potash is very much higher in NP(1.5K) without, however, reaching the level attained in 2(NPK). The ranges are from 7.80 to 6.00 percent for NP(1.5K) and from 6.59 to 4.35 percent for NPK. The forms of the graphs also are different. Potash accumulates in NP(1.5K) after the second period.

Related to these differences is superior development of the aerial parts and tubers in NP(1.5K).

Comparison of the Manure Plot (1–15) With the NPK Plot (1–10)

Nitrogen is lower on the manure plot at the first sampling date and higher at the last date than in NPK; the ranges are from 4.65 to 3.28 percent and from 4.98 to 3.14 percent, respectively.

Phosphoric acid is much higher throughout the period in the leaves from the manure plot; the ranges are from 0.69 to 0.45 percent and from 0.58 to 0.39 percent for manure and NPK, respectively.

Potash is higher at each date of sampling in the manure plot. The range is from 7.55 to 5.66 percent for manure and from 6.59 to 4.35 percent for NPK. Potash accumulation occurs during the last period (August 24) in the manure plot.

Related to these differences is a much greater development of the plants on the manure plots which is noticeable even on July 7 and which, with the exception of the second period, continues throughout the period. The yields of tubers per plant are 754 g in the manure plot and 466 g in NPK on August 24.

Note on the Development of the Plants

The development graphs of all plots are regularly progressive and show no indications of any interfering accidental factors outside of those studied. Plants on duplicate plots have not always developed in like manner. Between some of the duplicates there are marked differences in both aerial parts and tubers, suggesting the influence of soil on the action of fertilizers.

SUMMARY

Foliar diagnosis is defined as the composition, with respect to the dominant elements, of a leaf from a definite position (physiological age) on the stem at the moment of sampling. The foliar diagnosis of any particular species during a given season's growth cycle will consist, therefore, in a sequence of chemical states in the selected leaf as determined by periodic analysis. The composition with respect to nitrogen, phosphoric acid, and potash is based on the dry material of the leaf without taking into consideration either the weight or the number of leaves sampled.

The nitrogen, phosphorus, and potassium content of leaves of the same physiological age, sampled periodically, from potato plants

grown on plots receiving different fertilizer treatments with respect to nitrogen, phosphoric acid, and potash have been studied in relation to the nature of these treatments and to the development of the plants. The characteristics are fully described.

The addition of each of the dominant nutrient elements to the soil increased the content of the element in the leaves sampled, as compared with the content in leaves without applications of the element, whether the element was applied alone or in combination with one or both of the other elements. Utilization of any nutrient, however, was profoundly influenced by the presence in the fertilizer of other nutrients, as illustrated by accumulation of potash in leaves from plots receiving potash fertilizer but with insufficient nitrogen or phosphorus.

The content of nitrogen in the leaves is not related to the development of the plants; low phosphorus content and low utilization of this element are, however, associated with unsatisfactory development. Equilibrium between nitrogen and phosphorus throughout the season is associated with good development, as is also a maintained rapid utilization of potassium at a high level.

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OBSERVATIONS ON MUSKRAT DAMAGE TO CORN AND OTHER CROPS IN CENTRAL IOWA[1]

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INTRODUCTION

Despite the value of the muskrat (*Ondatra zibethica*) as a fur bearer, popular sentiment regarding the species is not always favorable. Many people believe that it causes economic losses because of its feeding or digging proclivities. In central Iowa, one of the chief sources of complaint against the muskrat is its occasionally destructive foraging in cultivated fields. Damage to farm crops by wild animals is likely to be overestimated, however, when it becomes conspicuous. It is hoped, therefore, that the present paper, which is based upon a study of muskrat food habits in intensively cultivated land, may help to a clearer evaluation of at least some types of local situations.

METHODS OF INVESTIGATION

Basic data on the food habits of muskrats were obtained from the summer of 1934 to the winter of 1937–38, largely through field observations along watercourses within a radius of 20 miles from Ames, Iowa, or from Story County and the neighboring counties of Hamilton, Boone, Dallas, and Polk; supplementary observations were also made in Wapello and Jefferson Counties in the southeastern part of Iowa and in Clay and Palo Alto Counties in the northwestern part.

Representative muskrat habitats[2] were chosen for study. These varied in nature from brooks and small drainage ditches to marshlands and the fairly large Des Moines River. Stream habitats under ordinarily intensive observation were visited at intervals sufficient to detect evident feeding trends, influence of seasonal changes in vegetation and water levels, and the comparative role of availability of food and individual preference in determining the feeding routine of muskrats. When events of exceptional interest or significance were found to be taking place, specific habitats were visited daily or several times a week for necessary periods; otherwise, habitats were seldom visited more than once a week. Pressure of work elsewhere or conditions unfavorable for field study sometimes necessitated temporary neglect of important habitats, but, on the whole, the same local areas were kept under observation during the 4 years of the investigation.

The general procedure was to take notes on vegetation cut or eaten by muskrats on stream banks, on the food debris piled on or scattered around feeding places in or near the water, and on the material carried to burrows which were sometimes exposed by receding water or caving

[1] Received for publication March 18, 1938; issued September 1938. Journal Paper No. J-532 of the Iowa Agricultural Experiment Station; project No. 498. The work herein recorded was conducted by Iowa State College in cooperation with the Bureau of Biological Survey, U. S. Department of Agriculture, the American Wildlife Institute, and the Iowa Conservation Commission.

[2] ERRINGTON, PAUL L. HABITAT REQUIREMENTS OF STREAM-DWELLING MUSKRATS. North Amer. Wildlife Conf. Trans. 2:411–416. 1937.

of the ground so that one could look inside with the aid of a flashlight. While this ex post facto "reading of sign" doubtless was accompanied by a certain amount of overlooking if not misinterpretation of field evidence, the accuracy of the method was checked whenever possible by other means. Occasionally, the examination of muskrat feces or stomach contents provided information of value, but most useful of all in clarifying dubious interpretations of "sign" was direct observation of the muskrats themselves.

Many times, muskrats were watched from distances of 15 to 40 feet when feeding or obtaining food. Details of procedure in selecting, cutting, digging, carrying, and eating of food items were recorded in field notes, and the sites of activity were often carefully scrutinized. On exposed feeding grounds still in use during the months of cold weather, snow trails of individual muskrats were followed and studied.

FORAGING TENDENCIES OF THE MUSKRAT

Muskrats, being animals of semiaquatic habitats, typically cut or feed upon aquatic or weedy vegetation growing in the vicinity of their retreats, but they may at times forage on higher ground some distance away.

Considerable feeding may occur in pasture growths of bluegrass and white clover, in timothy, sweetclover, and alfalfa hayfields, and in fields of small grain, but even when muskrat trails to such places show regular traffic it is often hard to find much evidence of damage. Ordinarily the animals forage lightly over an area of several hundred square feet, but, on the other hand, places may be found near the edges of fields where muskrats have cut away entire stands of medium weight vegetation from irregular areas ranging in size from less than a square foot to perhaps 15 or 20 square feet. Muskrats have also been known to make inroads on garden vegetables, but damage to farm crops in the areas with which this paper deals seems virtually synonymous with damage to corn.

Damage to corn may, too, be negligible or it may be locally severe. Much depends upon the accessibility of the cornfields to resident muskrats and the habits that the animals develop. Muskrats do not like to leave the relative safety of water very far behind, and this automatically limits their zone of heaviest potential damage to the nearest corn rows. Once a muskrat learns to utilize corn plants or ears, it usually prefers these foods to others currently available, and to obtain them may expose itself to material hazards. During the winter of 1934–35, one farm dog was reported to have killed several muskrats that had been traveling a distance of over 200 yards from a creek to a corncrib. When such overland journeys are made to a specific destination, the muskrats tend to establish crooked but well-used trails which afford them the concealment or protection of roadside ditches, low-hanging trees, brush piles, or similar cover. Nevertheless, corn rows within 15 yards of the water are most likely to receive regular visits from muskrats.

SEASONAL CHANGES IN HABITS OF CORNFIELD-FEEDING MUSKRATS

In winter, Iowa stream-dwelling muskrats are commonly confined rather closely to their burrows and associated water, ice shelves, and snowdrifts. With the passing of cold weather, early spring visits to

cornfields may be made either accidentally by wandering or venturesome individuals or seemingly with purposeful intent on the part of individuals that presumably have been there before. Ear corn may be found on the ground in some quantity—even after a field has been pastured and trampled by livestock—but less nutritious corn debris, including dry stalks, leaves, and husks, may also be carried to spring feeding places and eaten.

During the next few weeks, the natural plant growth near the water furnishes more convenient and attractive food, and there may be no sign of renewed activity in the cornfields until about the first week of July or later. By this time, the corn plants in central Iowa may be 3 or 4 feet high, and seem considerably more succulent and palatable than much of the natural vegetation available to the muskrats. The stalks are usually cut between 3 and 12 inches from the ground and, in places thought to be visited by lone muskrats, the rate of cutting has been only one or two per day. The whole upper part of the cut plant may be carried to a regular feeding place at the water's edge, or much feeding may be done in the cornfield at the site of cuttings. The canes seem especially relished by the muskrats and the upper leaves least so.

The corn plants that are about 6 feet high by the middle of July are cut in limited amounts, and haphazard or desultory gnawing may weaken a number of others and eventually cause them to fall. The interest of the muskrats in vegetative portions of corn plants sometimes diminishes in late July, and prior to the roasting-ear stage there may be scant evidence of current activity in the cornfields.

Roasting ears in the second half of August, however, may constitute a major attraction. While many muskrats take ears from leaning or fallen stalks or reach up as high as 15 inches for them, there may be wholesale cutting of stalks for ears by other individuals or groups of individuals. Commonly, the ears are cut loose at the base and carried away, though an occasional ear may be eaten from below as it hangs from its stalk. Aside from ears dropped in trails and in the water in front of the entrances of the burrows, there may be little external sign of most of the ears taken; but caved-in sections of burrows and similar retreats may reveal ears and mixed corn debris, so it is probable that the bulk of the material goes underground for safer consumption if not for actual storage. Ears found about muskrat retreats at this season, whether in plain sight on land or in water or retrieved from burrows or tile openings, show all stages of feeding, from specimens with most of the husks intact to those with not only husks and kernels gone but also large pieces of cob.

With the ripening of the corn in September, cutting of stalks for ears may persist locally, but according to observational data in hand, on a decreased scale. There may be continued heavy or accelerated traffic into the cornfields for ears on fallen stalks or lying free on the ground. From ripening time until the water courses freeze over, the most general foraging in cornfields is noted. Ripened ears, like those in the roasting-ear stage, are usually taken into the burrows. The evidence suggests that corn ears stored or accumulated in the burrows comprise a very important item in the winter diet of many muskrats, even after the material becomes soggy and stale.

After "freeze-up," some muskrats, for no known reason, persist in visiting cornfields long past the time that other muskrats have

ceased to come out, or they resume outside activities during thaws. Others, such as those living in burrows exposed by the drying up of marshes and stream beds, may through necessity and regardless of the weather, forage daily in the open, in which event cornfields may be particularly inviting to them. Still others may wander about the countryside, establishing themselves in farmyards, corncribs, corn shocks, or simply living where they can; muskrats thus abroad in midwinter find little in corn fields except dry stalks rejected by cattle, but they eat these in the absence of anything better.

During dry years, muskrats evicted from their accustomed habitats may actually improvise winter quarters in the cornfields. These quarters vary from flimsy temporary nests lined with dry grass and husks to large dry-land lodges constructed of stalks, weeds, mud, and other portable though substantial materials. One farmer reported finding about a bushel of ear corn in one cornfield habitation that he opened.

EVALUATION OF AN EXTREME INSTANCE OF MUSKRAT DAMAGE TO CORN

The most conspicuous damage from muskrat feeding that was noted during the investigation occurred along a half-mile stretch of drainage ditch southwest of Story City, Iowa.[3] Since destructive raiding into adjoining cornfields appeared to be a regular annual phenomenon, the ditch and environs were kept under careful observation throughout 1937.

In that year corn was planted in three fields bordering almost one entire side of the ditch; on the opposite side of the ditch was a closely grazed pasture, a field of mixed oats and barley, and a potato field. The ditch itself was about 5 feet wide at the bottom and about 8 feet below the level of most of the surrounding land, but it often contained only an interrupted series of shallow puddles without water enough for a steady flow. The dirt from excavation had been piled on both sides of the ditch in parallel ridges. The ditchbanks were fairly well protected from grazing by livestock, and the vegetation was principally of grasses with growths of Compositae, Polygonaceae, Cyperaceae, and miscellaneous weeds.

The spring breeding population of muskrats was not determined with certainty, but there were four main foci of activity along the half mile of ditch at two of which young animals or tracks and feces of young animals could be seen later in the season. Young muskrats appear to remain quite close to their natal localities after weaning,[4] so a resident breeding population of two, and perhaps four, pairs of adults seemed probable. Carcasses of 39 muskrats trapped in November 1937 from the ditch, and mostly from the half mile under observation, proved to be of those of 3 adult males, 2 adult females, and 34 young of the season.[5] By November, the population along the half mile of ditch was largely concentrated in two sets of burrows; according to the trappers, the more extensive set yielded about 20 muskrats without having been completely trapped out and the other

[3] NE¼ sec. 22, T. 85 N., R. 24 W., Story County.

[4] ERRINGTON, PAUL L., and ERRINGTON, CAROLYN STORM. EXPERIMENTAL TAGGING OF YOUNG MUSKRATS FOR PURPOSES OF STUDY. Jour. Wildlife Management 1 (3-4):49-61, illus. 1937.

[5] The Iowa muskrat investigations have brought forth no evidence of young muskrats becoming sufficiently mature to breed in the same season in which they are born. See the following: ERRINGTON, PAUL L. THE BREEDING SEASON OF THE MUSKRAT IN NORTHWEST IOWA. Jour. Mammal. 18:333-337, 1937; and the reference cited in footnote 4.

yielded a total of 7. This should indicate a pretrapping fall population close to 40 individuals, made up for the most part of the adults, and increase of from 2 to 4 pairs, if the sex and age ratios of the ditch-caught muskrats are representative of the animals resident along the half mile under observation. Incidentally, the ratio of young animals to adults given above is the highest recorded in the course of 4 years' study.

From March 7 to 16, 1937, evidence was found that muskrats had visited cornfields nearby and fed on ear corn at the water's edge. Some feeding on ear corn was noted as late as April 14, after which the animals seemed to find the cornfields less profitable foraging than the vegetation-grown ditchbanks. Thereafter, no sign of renewed activity in cornfields was detected until the new corn plants were of substantial size.

By July 14 and 15, considerable evidence of muskrats cutting down corn plants could be seen in the three cornfields bordering the half mile of ditch. At this time, the cutting was judged to have started more than a week previously in some places and less than a week in others. A counted total of 144 corn plants had been cut up to July 15. Between July 15 and 30, stalks were being cut at rates ranging from 6 to 11 per day and averaging about 2 per day for each of the four main foci of activity.

A marked increase of foraging in the cornfields occurred in mid-August and this had reached its apparent height by August 20. It is not clear whether this was due to the attractiveness of the ear corn, now at the roasting-ear stage, or to the adults having been joined in their foraging by the larger of the young. Some of the young were doubtless between 3 and 4 months old and nearly as large as small adults. In July, young animals were observed feeding chiefly on natural vegetation in or near the water, but there is no reason to believe that the larger ones were not venturing out to help themselves to the roasting ears. The technique of the muskrats in procuring the ears, however, seemed to be that of strong and experienced animals.

Most of the roasting ears were taken from the four main feeding areas where there had been cutting of corn plants earlier in the summer. In two of these, the muskrats took ears principally from leaning or fallen stalks and did little cutting; in the other two, cutting of stalks for ears was noticeable on an extensive scale and, on August 25, at the rate of about 40 per day. The total number of cut stalks counted up to August 20 was 1,304.

No thorough examinations of the affected cornfields were made from August 25 to September 19, but by the latter date, cutting of stalks for ears had greatly decreased with the ripening of the corn about 2 weeks before. Stalks evidently cut since ripening totaled 115. These added to an estimated 40 per day for perhaps 15 days subsequent to August 20, plus those cut before August 20, would give something like 2,000 cut stalks by September 19.

From this time on to November 4, the cutting and foraging of most of the muskrats was centered in an area in the corner of one of the cornfields. The area of intensive exploitation increased from 222 square yards on September 19 to 718 square yards by November 4; the few stalks left standing were estimated to be about the same in number as those cut outside, so, for practical purposes, the corn in the area cut over should be roughly the equivalent of that taken by the

muskrats. The increase of 496 square yards in cut area amounts to the destruction of 364 hills. One hundred eighty-two hills averaged 3.1 stalks, and this would give a total of 1,128 stalks cut by the muskrats since September 19, or an average of 24.5 per day for 46 days. Elsewhere along the half mile of ditch, muskrats visited the cornfields principally for ears on the ground, including those overlooked by men picking corn.

Subsequent damage was ascertained with less accuracy. The trapping season opened November 10, and the muskrats using the trails were soon caught. A few days later, the weather turned cold enough to put an end to the cornfield visits of the remaining muskrats for the rest of the fall. It seems reasonable to assume that the muskrats averaged 8 days of foraging after November 4. On the pro-rata basis of 24.5 stalks cut per day, about 188 additional stalks would be cut for ears before the death of the animals or the completion of the corn picking terminated destructive types of feeding activity in the cornfields.

To recapitulate: A total of about 3,300 cornstalks cut from July to the middle of November 1937, constituted the recognized muskrat damage to corn in three fields adjacent to the half mile of drainage ditch. Two hundred stalks examined at random in the fields bore 171 ears (including 18 nubbins), which would make the 3,300 cut cornstalks equivalent to the loss of over 2,800 ears of corn; at the average weight of 7.35 ounces per ear obtained from a random sample of 81 ears, 2,800 ears would represent a loss approaching 18.4 bushels of corn chargeable to the resident muskrat population.

The price of 1937 corn in Story County was $0.39 per bushel on November 15,[6] and fur buyers were offering locally about $0.60 flat rate for muskrat pelts. While the actual sums received from both corn and muskrat pelts would vary with circumstances and with the judgment of the sellers, at the above ratio of prices the $16.20 fur value of the 27 muskrats trapped is over twice the $7.18 known corn loss.

Even if one arbitrarily added $3 or $4 to the computed corn damage to cover the loss of ears taken by muskrats without cutting stalks—which really did not appear to have economic significance in this instance—the total damage to the corn would still be lower than the fur returns from the half mile of ditch. It is, of course, true that the farmers losing corn are not necessarily the persons to profit from the sale of the trapped pelts, though to do so is often within their choice.

DISCUSSION

During 1937, in particular, a concerted effort was made to evaluate muskrat damage to farm crops in the fields bordering about 11 miles of watercourses, exclusive of minor windings of channels. Dry weather in late summer and early fall evicted muskrat populations from some stretches of stream bed, but it is not thought that this reduced the number of animals enough to result in appreciable lessening of foraging in cornfields still accessible. On the contrary, the drought may be said to have intensified muskrat activity at increasing distances from their regular quarters.

[6] County agricultural agent reports.

Observations were made in 26 fields of corn within convenient reach of habitats occupied by muskrats in 1937. Of these, only the three adjacent to the half mile of drainage ditch described in this paper consistently suffered damage, but, in one other case, the autumn traffic for ears on fallen stalks was such that perhaps 30 or 40 ears could be seen at one time on the river bottom in front of a well-used system of burrows.

Twelve of the twenty-six cornfields showed evidence of light or very moderate use, and 10 were apparently not used at all by muskrats. Moreover, the great majority of central Iowa cornfields are not nearly so accessible to muskrats as the 26 under observation.

As a rule, foraging by muskrats occurs year after year in much the same local areas. Fields intersected by drainage ditches are particularly susceptible to damage by muskrats living in the vicinity; fields surrounding marshes are least troubled, possibly because of the abundance of favored foods usually present in natural marshy habitats.

SUMMARY

Data on food habits of muskrats in central Iowa were gathered from the summer of 1934 to the winter of 1937–38. Much the same areas were kept under observation during the entire period of study, and these were representative of intensively cultivated farm land adjacent to muskrat stream habitats of various types. Damage to farm crops through the foraging of muskrats was confined almost exclusively to corn in fields close to watercourses.

The heaviest damage to corn recorded during the investigation occurred in three fields adjacent to a half mile of drainage ditch in Story County. The situation here was observed in some detail throughout 1937, and the visible loss in yield because of muskrats was appraised at $7.18 (equivalent of 18.4 bushels at $0.39 as of November 15). The current market value of the muskrat pelts taken by trappers from corresponding parts of the ditch was $16.20 (27 pelts at $0.60 flat rate).

Of 26 cornfields bordering about 11 miles of muskrat-occupied watercourses in 1937, only 1, in addition to the 3 near the drainage ditch, showed clear evidence of damage; the other 22 either were not used at all by muskrats or were used so slightly that the losses were immaterial.

TOXIC AND REPELLENT PROPERTIES OF SOME INORGANIC AND ORGANIC COMPOUNDS AND MIXTURES IN GRASSHOPPER BAITS [1]

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INTRODUCTION

This investigation forms a part of a project which has continued in Iowa for a number of years, some of the results having been published in 1932 (*9*) [3] and 1933 (*10*). The practical objective has been a poison less toxic to human beings and domestic animals than sodium arsenite yet equally effective on grasshoppers, readily available, and low in cost. Attention has also been given to repellent action and to other phenomena of toxicological interest, for it is recognized that a compound even mildly toxic or repellent to such resistant insects as grasshoppers might be valuable in the control of less resistant species.

Previous studies in this series were concerned with the rather precise estimation of the dosage of a few poisonous compounds; in this investigation, an attempt has been made to survey the toxic and repellent properties of a much larger number of inorganic and organic chemicals.

The experiments were made at Ames, Iowa, during the period from June 1 to August 12, 1937.

MATERIALS

One hundred and ten compounds and mixtures were tested in bran-water baits during the season. Unless otherwise stated in table 1, all the inorganic compounds were of chemically pure or reagent grade. Some of United States Pharmacopoeia grade are so indicated. Most of the organic chemicals were of the highest grade obtainable from reliable manufacturers. Some, however, were of a technical grade as shown in the table. The nature of the mixtures is described briefly in footnotes.

The bran, which formed about half the weight of the finished bait, was a clean, flaky, commercial product milled from wheat.

The test insects were adults of the lesser migratory grasshopper (*Melanoplus mexicanus mexicanus* Saussure). They were captured in western and southern Iowa, kept in large cages, and fed daily on fresh corn foliage. The insects to be used in the tests were held in the laboratory overnight without food.

[1] Received for publication March 18, 1938; issued September 1938. Journal Paper No. J-536 of the Iowa Agricultural Experiment Station; project No. 137.

[2] The writers are indebted to L. E. Mills of the Dow Chemical Co., to Dr. J. L. Horsfall of the American Cyanamide Co., to Dr. R. C. Roark of the Bureau of Entomology and Plant Quarantine, and to Dr. W. H. Tisdale of DuPont de Nemours & Co. for certain chemicals; and to Jean Austin for able assistance.

[3] Italic numbers in parentheses refer to Literature Cited, p. 432.

METHOD

The bait consisted of 10.0 g of bran, 9.8 cc of distilled water, and 0.2 g of the compound or mixture, the concentration of the latter ingredient being therefore 1 percent by weight. Insoluble solids, if not already in a finely divided condition, were ground to a fine powder before they were incorporated in the bait. Water-soluble substances were dissolved in the water before it was mixed with the bran. The bait was held in covered containers which were opened only when a quantity of it was removed for the tests.

The method of administering the bait was modified somewhat from that previously described (*9*), for it was found that *Melanoplus m. mexicanus* was more active than the species hitherto employed, frequently upsetting the bait containers when attempts were made to feed the insect in large moist chambers. The modification consisted in weighing about 0.5 g of the moist bait in a small, tared container made of paraffined paper with a hole in one edge by which it was suspended from a hook on the beam of the balance. The container was thrust toward the stoppered end of a large glass tube which was always maintained in a horizontal position. A narrow strip of screen wire provided the tube with a floor which caught and held the bait particles sometimes spilled by the insect. A previously weighed grasshopper was carefully introduced into the open end of the tube which was then closed with a cork stopper. A wad of moist cellucotton, fastened to this stopper, supplied moisture to the air in the tube. The change in moisture content of the bait during the feeding period was determined, and found to be within the limits of precision of the method. When feeding ceased, the insect was removed to a small screen cage where it was observed at intervals until death; or, if apparently unaffected, for a period of 72 hours.

Two standard baits were administered throughout the experimental period to grasshoppers taken from the same populations that furnished individuals for the other tests. The standard baits were: (1) A bran-water bait (10.0 g of bran and 10.0 cc of water) on which 50 insects were fed during the period; (2) sodium arsenite bait (10.0 g of bran and 10.0 cc of an aqueous solution containing 0.2 g of As_2O_3 as NaH_2AsO_3) fed to 66 insects during the same period. The latter mixture contained approximately the same concentration of sodium arsenite calculated as As_2O_3 as that employed in Iowa during the grasshopper-control campaign of 1937. Molasses was not used in these baits; it is now practically excluded from the baits employed in large-scale poisoning in the Middle West.

A test consisted of 10 grasshoppers fed individually, of which 5 were males and 5 females in order to equalize possible sex differences. Since about 1 in each sample of 10 control insects fed the bran-water standard died from natural causes,[4] the test was usually repeated on another sample of 10 insects if 2 or more individuals died in the first sample.

The criteria of effect of the added compounds or mixtures were: (1) Net mean mortality obtained from the formula $M=(x-y)100/x$, in which x represents the mean percentage of insects alive 72 hours

[4] The actual mortality for the control insects was 12 percent. In 312 insects fed fresh corn foliage during the experimental period, a smaller proportion, 5.4 percent, died of natural causes. Normal mortality was, therefore, moderate in the populations from which the test insects were drawn.

after feeding ad libitum on the bran-water standard, and y the mean percentage alive after feeding on the experimental bait; (2) mean survival time, the mean interval in hours from cessation of feeding until death, death being the failure of the insect to respond by movement to pinching of the tarsi or palpi with forceps; (3) mean bait consumption, the mean quantity in grams of bait eaten; (4) mean feeding time, the mean interval in minutes from beginning to cessation of feeding; and (5) milligram/gram, the mean quantity of compound or mixture in the consumed bait calculated as milligrams per gram of body weight of the insect.

THEORETICAL COMBINATIONS OF MORTALITY, BAIT CONSUMPTION, AND FEEDING TIME

In a series of data based on mortality with respect to a nontoxic food substance, there are only two combinations involving mortality, namely, a series without mortality and a series in which mortality is present. On the other hand, data on bait consumption from insects fed ad libitum may be grouped into three combinations expressing levels of bait intake with respect to the standard; that is, consumption higher, the same, and lower than that of the standard. Data on feeding time from insects fed in the same manner, likewise, may be placed in three groups; namely, time longer, the same, and shorter than that on the standard. There are then 2 possible mortality series and 9 possible combinations within each series, or a total of 18 theoretical combinations of mortality, bait consumption, and feeding time.

In the tabular arrangement of these theoretical combinations, given below, M indicates mortality, C bait consumption, and T feeding time, whereas the levels are indicated by h, s, and l, signifying, respectively, greater than, the same or similar to, and less than the standard.

Mh, Mortality greater than that of the standard food substance:
- *ChTh*, Very attractive toxic baits; eaten slowly.
- *ChTs*, Very attractive toxic baits; eaten in normal time.
- *ChTl*, Very attractive toxic baits; eaten voraciously.
- *CsTh*, Not markedly distasteful toxic baits; eaten slowly.
- *CsTs*, Toxic baits; consumption and feeding time like standard.
- *CsTl*, Toxic baits; consumption normal, but eaten rapidly.
- *ClTh*, Repellent toxic baits; eaten slowly.
- *ClTs*, Toxic baits; consumption small, but eaten in normal time.
- *ClTl*, Toxic baits; consumption small, but eaten in a short time.

Ms, Mortality not greater than that of the standard food substance:
- *ChTh*, Very attractive baits; eaten slowly.
- *ChTs*, Very attractive baits; eaten in normal time.
- *ChTl*, Very attractive baits; eaten voraciously.
- *CsTh*, Attractive baits; consumption normal but eaten slowly.
- *CsTs*, Attractive baits; consumption and feeding time like standard.
- *CsTl*, Attractive baits; consumption normal, but eaten rapidly.
- *ClTh*, Repellent nontoxic baits; consumption small, but eaten slowly.
- *ClTs*, Repellent nontoxic baits; consumption small, but eaten in normal time.
- *ClTl*, Repellent nontoxic baits; consumption small, but eaten in a short time.

RESULTS

The results are arranged in table 1 according to the combinations just discussed. Compounds which gave net mean mortalities of less than 15 percent are considered doubtfully toxic at the concentration employed.

TABLE 1.—*Effect on grasshoppers of compounds and mixtures in bran baits*

Compound or mixture in bait	Theoretical combination [1]	Mean bait consumption [2]	Mean feeding time [2]	Milligram/gram [2]	Mean survival time	Net mean mortality
		Grams	*Minutes*		*Hours*	*Percent*
Bran-water standard (no added chemical substance).		0.038	17			100
Zinc phosphide (98.7 percent Zn_3P_2)	*MbChTh*	**.055	**34	1.864	22	77
Thallous sulphate	*MbCaTh*	.046	**25	1.175	36	100
Ammonium fluoride	*MbCaTs*	.030	13	1.102	13	100
Potassium fluoride ($KF.2H_2O$)	do	*.049	15	1.641	13	100
Arsenic trioxide	do	.020	19	.822	37	77
Arsanilic acid	do	.039	20	1.082	42	15
Mercurous chloride	do	.030	18	.891	47	20
Crystal violet (dye content 94 percent)	do	.033	20	1.061	42	20
Diphenyl	do	.038	20	1.012	31	100
Ammonium bifluoride ($NH_4F.HF$)	*MbCsTl*	.028	**10	1.013	15	100
Zinc arsenite (tech.)	*MbClTs*	**.022	14	.715	47	93
Sodium arsenite (NaH_2AsO_3)	*MbClTl*	**.014	**8	.447	31	0
Magnesium carbonate	*MsChTh*	**.060	**38	2.270	.....	0
Sodium benzoate	do	**.052	**38	1.890	.....	0
Zinc cyanamide (tech.)	do	**.059	**34	1.874	.....	0
Barium fluoride	*MsChTs*	**.057	19	1.928	.....	0
Calcium fluoride	do	**.057	*22	1.745	.....	0
Chromic oxide	do	**.052	17	1.619	.....	0
Antimony pentoxide	*MsCsTh*	.037	**34	1.201	.....	0
Antimony potassium tartrate (U. S. P.)	do	.046	**24	1.230	.....	0
Bismuth trioxide	do	.041	**25	1.152	.....	0
Lead benzoate	do	.044	**31	1.554	.....	0
Lead chromate	do	.038	**24	1.104	.....	3
Magnesium acetate [$Mg(C_2H_3O_2)_2.xH_2O$]	do	*.048	**24	1.566	.....	0
Potassium permanganate	do	*.052	**24	1.562	.....	0
Stannous chloride ($SnCl_2.2H_2O$)	do	.038	**23	.976	.....	3
Acetone sodium bisulphite	do	.036	**31	1.101	.....	0
Hexachlorobenzene	do	.034	**25	.930	.....	0
Triphenylmethane	do	*.048	**30	1.227	.....	0
Triphenylguanidine	do	.044	**30	1.219	.....	0
Ammonium oxalate	*MsCsTs*	.029	20	.815	.....	0
Antimony trioxide	do	.045	16	1.043	.....	0
Barium chromate	do	.038	21	1.139	.....	0
Bismuth oxychloride	do	.033	16	.967	.....	0
Bismuth subcarbonate	do	.044	21	1.137	.....	0
Bismuth subnitrate	do	.039	20	1.164	.....	0
Boric acid	do	.037	*22	1.246	.....	0
Cadmium chloride	do	.034	16	1.054	.....	3
Cadmium nitrate [$Cd(NO_3)_2.4H_2O$]	do	.039	19	1.149	.....	0
Cadmium oxide	do	.038	20	1.099	.....	0
Cadmium sulphate ($3CdSO_4.8H_2O$)	do	.033	16	.992	.....	0
Calcium carbonate	do	.043	16	1.179	.....	0
Lead borate [$Pb(BO_2)_2.H_2O$]	do	.042	*25	1.244	.....	0
Magnesium chloride ($MgCl_2.6H_2O$)	do	.032	16	1.311	.....	0
Magnesium citrate	do	*.049	21	1.564	.....	0
Magnesium fluoride	do	.041	*23	1.075	.....	0
Magnesium oxide	do	.035	*23	.954	.....	0
Magnesium oxalate ($MgC_2O_4.2H_2O$)	do	.042	20	1.113	.....	0
Magnesium phosphate [$Mg_3(PO_4)_2.4H_2O$]	do	.032	22	.791	.....	0
Magnesium sulphate, 12 percent (U. S. P.) ($MgSO_4.7H_2O$).	do	.028	15	11.154	.....	0
Manganous sulphate ($MnSO_4.2H_2O$)	do	.039	19	1.099	.....	0
Potassium bromate	do	.040	18	1.155	.....	9
Potassium chrome alum	do	.039	20	1.092	.....	0
Potassium ferricyanide	do	.034	22	1.078	.....	0
Potassium ferrocyanide [$K_4Fe(CN)_6.3H_2O$]	do	*.050	20	1.423	.....	0
Potassium iodide	do	.047	21	1.230	.....	0
Potassium methyl sulphate	do	*.053	22	1.544	.....	0
Sodium iodate	do	.028	*11	.652	.....	0
Sodium lauryl sulphate	do	.028	21	.785	.....	0
Sodium sulphide ($Na_2S.9H_2O$)	do	.030	14	.931	.....	9
Sulphur	do	.044	16	1.207	.....	0
Crotonic acid	do	.033	20	1.173	.....	0
Trithiomethylene	do	*.051	17	1.860	.....	0
Copper cyanamide (tech.)	do	.035	25	1.034	.....	9
p-Aminophenol	do	.039	17	1.097	.....	0
o-Cyclohexylphenol	do	.037	21	1.136	.....	0
p-Cyclohexylphenol	do	.046	*22	1.440	.....	0
Sodium phenolsulphonate (U. S. P.)	do	.032	*11	.848	.....	0
Tri-*o*-cresylphosphate	do	.037	14	.979	.....	3
Resorcinol (U. S. P.)	do	.037	21	1.103	.....	0
α-Naphthol	do	.027	22	.734	.....	0

See footnotes at end of table.

TABLE 1.—*Effect on grasshoppers of compounds and mixtures in bran baits*—Continued

Compound or mixture in bait	Theoretical combination	Mean bait consumption	Mean feeding time	Milligram/gram	Mean survival time	Net mean mortality
		Grams	*Minutes*		*Hours*	*Percent*
β-Naphthol	*MsCsTs*	0.029	19	0.849		0
XXX Phenols [4]	do	*.028	*23	.817		0
p-Nitrodiphenyl	do	.031	22	.883		9
p-Phenetidine	do	.026	*11	.771		0
m-Dinitrobenzene	do	*.024	13	.579		0
Safranine bluish	do	.029	21	.722		0
Settled wood tar [4]	do	.030	16	.751		0
Sulphanilic acid	do	.044	18	1.085		0
Thiocarbanilide	do	.043	20	1.239		0
m-Phenylenediamine	do	.040	*23	1.134		0
Diphenylguanidine	do	.031	17	.920		0
α-Naphthylamine	do	.029	16	.735		0
β-Naphthylamine	do	.042	15	1.527		0
Diphenylamine sulphate	do	.036	16	1.184		0
Piperidinium tetrathiocyanatodiammino chromium.	do	.046	*21	1.222		0
Magnesium sulphate, 1 percent (U. S. P.)	*MsCsTl*	.034	**10	.972		0
Paraformaldehyde	do	.030	**10	1.006		0
Quinoidine [5]	*MsClTh*	**.023	**27	.718		0
Barium thiosulphate ($BaS_2O_3.H_2O$)	*MsClTs*	**.024	20	.732		0
Cupric borate	do	**.022	12	.618		0
Cupric carbonate	do	**.021	*11	.532		0
Cupric sulphate ($CuSO_4.5H_2O$)	do	**.022	11	.618		0
Sodium thiocyanate	do	**.023	16	.618		0
Phenol	do	**.012	14	.363		0
Tetrachlorophenol (tech.)	do	**.016	15	.385		0
Pentachlorophenol (tech.)	do	**.024	15	.689		0
Benzamide	do	**.015	21	.516		0
Acridine	do	**.023	13	.712		0
p-Nitrophenetole	do	**.022	19	.715		9
Nicotine peat [6]	do	**.016	18	.444		0
Cupric acetate [$Cu(C_2H_3O_2)_2.H_2O$]	*MsClTl*	**.013	**8	.400		0
Mercuric chloride	do	**.010	**6	.295		0
Magnesium sulphate, 35 percent (U. S. P.)	do	**.009	**5	7.666		0
3,5-Dinitro-o-cresol	do	**.011	**4	.299		3
Magnesium 2,4-dinitro-6-cyclohexylphenate	do	**.008	**5	.214		0
Acetophenone	do	**.009	**4	.260		0
XXX Oil [4]	do	**.022	**9	.568		0
p-Dichlorobenzene	do	**.016	**7	.452		3
Naphthalene	do	**.022	**8	.609		0
o-Nitroaniline	do	**.008	2	.247		0
Isoquinoline	do	**.009	**8	.312		3

[1] *M* signifies net mean mortality, *C* bait consumption, *T* feeding time; and with respect to a nontoxic food substance, *h* indicates greater than, *s* the same as or similar to, and *l* less than.
[2] ** Signifies highly significant ($P=0.01$); * signifies a significant difference ($P=0.05$).
[3] Milligrams of added chemical per gram of body weight of the insect.
[4] From destructive distillation of wood.
[5] Mixture of amorphous alkaloids from cinchona bark.
[6] Peat containing 10 percent of total nicotine; 90 percent of the nicotine is insoluble in water when the aqueous mixture contains about 0.5 percent of nicotine peat.

Survival times are approximate. In some instances they could be determined closely; in others, particularly when the insect died during the night, the survival time was estimated. The data are valuable, however, when large differences are concerned.

The mean bait consumption for each experimental bait was tested by the *t* test for a significant deviation from mean consumption of the bran-water standard. Since the samples were small, only means which gave values for *t* corresponding with probabilities of 0.01 or less are considered significant. These are indicated in the table by two asterisks. Means that yielded *t* values corresponding with probabilities of 0.05 or less, but greater than 0.01, although usually regarded as significant in biological experiments, are not so considered in the interpretation of these results. They are marked by an asterisk

in the table. All values for bait consumption not so marked, unless qualified in the discussion following, are interpreted as probably equivalent or similar to mean consumption of the bran-water standard.

Feeding time is more variable, and its precise determination therefore more difficult, than that of bait consumption. Feeding time is subject to several errors. To avoid disturbance, the feeding insect must not be observed too closely. During long feeding intervals the insect may stop eating for a time, although from its position before the bait container it may appear to be feeding actively. Whenever such pauses were noted they were timed, and the time was deducted from the total feeding time. But it was not possible to detect them all; consequently, long feeding times are subject to greater error than short times. On cool days, the insects fed more slowly than on warm days. During cool weather, experimentation was usually discontinued, or the data were marked for later qualification. Again, certain compounds may have so affected the texture or other physical properties of the bait that the rate of feeding was reduced. Notwithstanding these inaccuracies, feeding time has been of considerable value in the interpretation of the action of certain chemicals. The data were tested for significant deviations from the bran-water standard in the manner described for bait consumption, and the means in table 1 are marked by asterisks in the same way.

DISCUSSION

The conclusions regarding the effects of the chemical substances on mortality, bait consumption, and feeding time in this discussion apply strictly to the concentration employed, namely, 1 percent by weight of the moist bait. The only exception was magnesium sulphate which was tested at 1-, 12-, and 35-percent concentrations.

Lethal group.—Only 12 of the 110 compounds and mixtures tested were significantly lethal. The lethal compounds are assigned to six of the nine possible combinations of bait consumption and feeding time.

MhChTh.—Only one compound, zinc phosphide, can be placed in this group. Apparently *Melanoplus m. mexicanus* resembles *M. differentialis* (Thos.) in survival time and susceptibility to this compound (*10*). In comparison with sodium arsenite on *mexicanus*, survival time is shorter, but the quantity of bait eaten and the feeding time are much greater; from arsenic trioxide it differs in the significantly shorter survival time and the larger dose (milligram/gram). The higher bait consumption and longer feeding time compared with those for the standard bait are believed of actual significance.

MhCsTh.—A single compound, thallous sulphate, is placed in this group. Although the survival time is similar, its toxicity is lower than that for sodium arsenite. The long feeding interval may be the result of overtiming; however, the presence of this poison does not significantly affect the quantity of bait consumed.

MhCsTs.—Seven of the twelve toxic compounds are assigned to this category. Ammonium fluoride and potassium fluoride have the shortest mean survival times (13 hours) of any of the toxic compounds. The survival time for arsenic trioxide is about equal to that for sodium arsenite, and probably equivalent to the survival times of

the other compounds in this category, except the two fluorides. Arsanilic acid ($p\text{-}H_2NC_6H_4AsO_3H_2$) is apparently less toxic than the other arsenic compounds used in this investigation. Brinley (*1*) tested a number of aromatic compounds of arsenic on the eastern tent caterpillar (*Malacosoma americana* (Fab.), none of which proved more toxic than the inorganic arsenates. Mercurous chloride was perhaps slightly toxic though certainly not repellent, whereas mercuric chloride (cf. *MsClTl*) was so highly repellent that little of the bait was eaten. Crystal violet, a triphenylmethane dye, which Campbell (*2*) reported highly toxic by mouth and by injection to silkworms (*Bombyx mori* L.), and diphenyl were just slightly toxic at the concentration used.

It is of interest that more than half the baits containing toxic compounds were eaten in quantities and in a time equal or similar to that of the bran-water standard.

MhCsTl.—Only ammonium bifluoride falls in this category. In toxicity, survival time and bait consumption, the data are not distinguishable from those for the two fluorides in the group above. But the feeding time is shorter. Additional information would possibly change the group locations of these fluorides.

MhClTs.—Zinc arsenite, the single compound in this category, resembled sodium arsenite in toxicity and bait consumption, yet the feeding time was longer.

MhClTl.—Sodium arsenite is the only compound which can be assigned to this category. On the basis of As_2O_3 concentration, it was slightly less toxic than As_2O_3 itself, but the difference is probably of no significance. The survival time was similar to that of the other arsenic compounds tested, except zinc arsenite, and significantly longer than the survival times of the soluble flourides. However, bait consumption was the smallest, feeding time the shortest of the toxic compounds examined. The results appear to support a former conclusion from a study of the effect of sodium arsenite on *Melanoplus femur-rubrum femur-rubrum* (DeG.) and *M. differentialis* that "the grasshoppers were not repelled by the odor or taste of the bait mixtures used, and ceased to feed only when they became satiated or were too sick to eat more" (*9, p. 1078*).

Nontoxic group.—Eight of the nine theoretical combinations were realized. The only combination to which a compound could not be assigned was MsChTl. Such a compound, so definitely attractive that large quantities of the bait containing it would be devoured in a short time, would seldom be found among substances selected for their toxic or repellent action.

MsChTh.—Three compounds are assigned to this category. The attractiveness of zinc cyanamide, a technical product, may have resulted from impurities. The apparent attractiveness of magnesium carbonate and sodium benzoate cannot, at present, be related to their physical or chemical properties.

MsChTs.—The three compounds in this category appeared to stimulate increased bait consumption. Barium and calcium flourides, relatively insoluble compounds, are apparently without toxicity to the insect used.

MsCsTh.—Twelve compounds, grouped here, showed significantly long feeding times. Although overtiming may have been frequent in this group, mean feeding times of 30 minutes or more probably have

actual significance. Some of these compounds may have rendered the bait somewhat distasteful, or have changed its physical properties with a resulting increase of feeding time; but direct observations are not available on these points. Lead chromate and stannous chloride were perhaps very slightly toxic at the concentrations used.

MsCsTs.—About half the nontoxic compounds fall in this category, in which there are no striking differences from the bran-water standard. A few compounds in this group gave net mortalities as high as 9 percent, and a few showed significant differences from the standard in bait consumption and feeding time that in larger samples might become highly significant. Noteworthy is the almost complete lack of toxicity of the cadmium compounds. Several investigators (*4*, *5*) have found certain cadmium compounds toxic to a number of species of insects. The seven magnesium compounds proved to be without toxicity, a result anticipated from the conclusions of several workers which were published while this investigation was in progress (*3*, *6*, *11*). Like the barium and calcium fluorides, the insoluble magnesium fluoride is nontoxic. Diphenylguanidine, reported by Ginsburg and Granett (*5*), to be quite toxic and repellent to silkworms, was without effect on *Melanoplus m. mexicanus* at the lower concentration employed in these tests.

MsCsTl.—Two compounds are assigned to this category. Magnesium sulphate at 1-percent concentration gave a short feeding time, whereas at 12-percent, the feeding time was normal.

MsClTh.—Quinoidine, a mixture of amorphous alkaloids from cinchona bark, evidently rendered the bait so unattractive that it was nibbled over a long feeding period. It is possible the actual feeding time was shorter than that given. This substance is reported as somewhat toxic though not repellent to the silkworm (*5*).

MsClTs.—There are 12 compounds in this group. Bait consumption was significantly low, and the feeding times for most of the compounds were shorter than that for the standard, although the differences are not significant. Three of the five copper compounds examined appear in this group. Larrimer (*8*) found cupric sulphate the most repellent of a number of substances for several species of grasshoppers and crickets. Pentachlorophenol, reported as rather toxic though not repellent to the silkworm (*5*), proved rather repellent and not toxic to *Melanoplus m. mexicanus* at the concentration used in these tests.

MsClTl.—Eleven compounds, all highly repellent, fall in this group. Only 2 of them, mercuric chloride and magnesium sulphate, the latter fed at the enormous concentration of 35 percent, are inorganic compounds. The results from magnesium 2,4-dinitro-6-cyclohexylphenate are in accord with those obtained by Kagy (*7*) with the calcium salt of this compound on *Melanoplus f. femur-rubrum*. The calcium salt was so distasteful to the latter insect that attempts to feed a lethal dose were futile, whereas the phenol itself was more than twice as toxic as arsenic trioxide. Although isoquinoline proved somewhat toxic and strongly repellent to silkworms (*5*), it was at most very slightly toxic and strongly repellent to *M. m. mexicanus* at 1 percent.

The data for the toxic compounds were examined for sex differences. The 4 survivors of the 66 insects that ate the sodium arsenite bait were females; and the females consumed a larger absolute quantity

of this bait, and a larger quantity relative to the body weight than the males. The difference between the means of bait consumption for the sexes was significant, but not highly significant. The means for survival time and feeding times were not significantly different. Nine of the fourteen insects that survived the diphenyl bait were females, and the size of the dose in milligrams/gram consumed by the females was highly significant, in fact, nearly twice that eaten by the males. No other differences in the effect of the toxic compounds on the sexes were as clearly suggestive of a significant sex difference as these.

The insects fed the bran-water bait consumed, before they ceased to eat, 0.10 g of the bait per gram of body weight. No significant sex differences were found in the quantity consumed relative to body weight. On the basis of a mean bait consumption of 0.038 g., and a mean body weight for both sexes of 0.384 g., these grasshoppers consumed approximately 10 percent of their weight at one feeding. This figure is smaller than the value (16 percent) obtained for the consumption of a bran-molasses-water bait by *Melanoplus f. femurrubrum* (*9*).

SUMMARY

One hundred and ten inorganic and organic substances were fed individually in bran-water baits to adult lesser migratory grasshoppers (*Melanoplus m. mexicanus* Saussure), the chemical substance constituting 1 percent by weight of the moist bait. The insects were allowed to feed ad libitum on the bait mixture. The net mean mortality within 72 hours, mean survival time in hours, mean bait consumption in grams, mean feeding time in minutes, and mean quantity of chemical substance consumed with the bait in milligrams per gram of body weight of the insect were recorded. The theoretical combinations of mortality, levels of bait consumption, and levels of feeding time with respect to a nontoxic food substance are tabulated and the results for each bait are grouped and discussed under the appropriate combination. Conclusions are limited strictly to the effects produced by the chemical substances at 1-percent concentrations.

The lethal compounds are grouped as follows: (1) Highly toxic compounds (93- to 100-percent mortality)—zinc phosphide, ammonium fluoride, ammonium bifluoride, potassium fluoride, arsenic trioxide, sodium arsenite, and zinc arsenite; (2) moderately toxic compounds (77-percent mortality)—arsanilic acid and thallous sulphate; (3) slightly toxic compounds (15- to 20-percent mortality)—crystal violet, diphenyl and mercurous chloride. The three fluorides gave the shortest survival times, zinc phosphide was intermediate with 22 hours, and the remaining lethal compounds ranged from 31 to 47 hours. Highest bait consumption in the lethal group was given by zinc phosphide, lowest by sodium arsenite and zinc arsenite. The insects fed longest on baits containing zinc phosphide, but only a brief period on the ammonium bifluoride and sodium arsenite baits. Sodium arsenite is outstanding in the small quantity of poison relative to body weight required for high toxicity.

The nontoxic substances varied greatly in their effect on bait consumption and feeding time. Consumption was greatest of baits containing magnesium carbonate, sodium benzoate, zinc cyanamide,

barium fluoride, and chromic oxide, and smallest of baits containing a rather large group of compounds among which were most of the copper compounds and many of the phenols used in these tests. Feeding time was abnormally long, or equal to that on the bran-water standard, on compounds that showed high bait consumption; but it was also very long on some chemicals that were consumed in normal quantities. Some of these long feeding times may have resulted from the effect of the compound on the texture or other physical property of the bait. All the cadmium and magnesium compounds were ineffective except magnesium sulphate, which was repellent at the enormous concentration of 35 percent. The insoluble fluorides of barium, calcium, and magnesium were nontoxic. Mercuric chloride was so repellent that little of the bait containing it was eaten.

Sodium arsenite and diphenyl are probably more toxic to the males than to the females.

If undisturbed, this grasshopper consumes about 0.10 g of a nontoxic bran-water bait per gram of body weight, or 10 percent of its weight at one feeding.

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PROTEOLYTIC ACTIVITY IN RELATION TO THE BLACKENING OF POTATOES AFTER COOKING [1]

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INTRODUCTION

In a previous paper (*15*)[2] a general survey was made of the factors related to the development of gray to black discoloration in boiled potatoes (*Solanum tuberosum* L.). Evidence was there presented which indicated instability of the protein in abnormal tubers, and it was concluded that this condition is associated with the accumulation of tyrosine, tryptophane, and other amino acids. Other observers of such accumulation (*8*, *11*, *13*) noted its correlation with a deficiency of potassium in plant tissue. Thus the nutritional and enzymic aspects of plant existence become associated, and it is apparent that abnormal activity of enzymes may follow upon malnutrition. Hartt (*8*) concluded that the accumulation of amino acids, which she observed in potassium-deficient sugarcane, was the combined result of diminished synthesis and translocation of protein. The present paper covers an investigation of proteolytic activity as a causal factor in the blackening of cooked potatoes. Since the early paper of Vines (*16*) mentioning the presence of "tryptase" in the potato, little data have been reported with reference to the proteolytic enzymes of the potato tuber.

Since most of the protein of potato tubers is soluble in the sap (*2*) it appeared advisable to use sap as the substrate. The proteolytic activity was determined by following the rate of liberation of amino acids during the autolysis of tuber tissue. As tyrosine seemed to be a primary factor in discoloration, particular attention was given to the release of this constituent. Potato tubers rate high in tyrosinase activity and, consequently, that enzyme must be inhibited; otherwise, much tyrosine may be lost through oxidation. Most of the inhibitors of tyrosinase are known to activate certain plant proteases, so that their use might introduce complications. Hence it appeared advisable to allow autolysis to proceed under anaerobic conditions.

Another reason for the choice of this treatment arose from the work of Paech (*12*) on autolytic decomposition of proteins in green tissues. He found that the activity of protease was readily modified by atmospheric oxygen after cell destruction, and that this oxidation was preliminary to activation. Since oxygen tension within the tuber is quite low, exclusion of air would approach the natural condition of the tissue in this respect.

[1] Received for publication March 19, 1938; issued September 1938. This work was supported by grants from the Wisconsin Alumni Research Foundation, University of Wisconsin.

[2] Italic numbers in parentheses refer to Literature Cited, p. 440.

PROCEDURE

Representative samples of normal and of abnormal tubers were washed, wiped carefully, and frozen with solid carbon dioxide. While still thoroughly frozen the samples were ground in a chilled meat grinder. Portions of the well-mixed tissue were placed in cooled flasks and an equal weight of oxygen-free water was added. Toluene was added as a preservative. In some cases twice the above weight of water was used. The flasks were fitted with rubber stoppers through which passed inlet and outlet tubes. Nitrogen gas, free of oxygen, was then bubbled through for several minutes. After the flasks were sealed they were allowed to stand (with occasional shaking) at room temperature for several hours. They were then placed in an ice box overnight to obtain complete thawing and exosmosis of cell contents, with a minimum of enzymatic change. During the next morning the digests were allowed to come to room temperature and samples were taken. After this they were incubated at 37° C. and sampled at intervals.

While the samples were being taken nitrogen was bubbled through the suspensions at such a rate as to provide thorough stirring. Portions were removed by means of a 50-cc pipette (with tip enlarged) and immediately added to 1 cc of 50-percent trichloroacetic acid. The preparations were mixed, allowed to stand one-half hour and then centrifuged. This procedure usually resulted in a clear, slightly amber-colored solution. Aliquot parts were taken from this for analysis.

To lessen the possibility that other variables than proteolytic activity might be included in this experimental treatment, the tubers selected for each test were of practically the same size and of the same variety. Analyses had proved that samples so selected show little or no consistent differences in hydrogen-ion concentration, moisture, or total protein content, or in the amount of protein in the prepared digests. The average results from several different tests should, therefore, be dependable. As the hydrogen-ion concentration of the digests remained practically constant during digestion, it was unnecessary to employ buffers.

A difference in the rate of hydrolysis during autolysis might equally well be due to either inequality in the ease of cleavage of proteins or variations in enzymic activity. As a test of these alternatives the enzyme concentration was increased by the addition of papain. The equivalent of 0.2 g per 100 g of tissue was added from a suspension in water of a commercial preparation of the enzyme. Controls were prepared by substituting an equal volume of water for the enzyme suspension. In other digests the amount of protein was increased by the addition of either potato protein (from normal tubers) or gelatin, to the extent of 2 percent of the tissue.

In certain tests the concentration of potassium in digests of abnormal tubers was made equal to, or in excess of, that of normal tubers, by the addition of 0.2 g of potassium in the form of potassium chloride. In some experiments sodium chloride was added to the autolysates (7.5 g per 100 g of tissue) for the purpose of possibly increasing the concentration of soluble protein.

DETERMINATION OF TYROSINE AND OF TRYPTOPHANE

An aliquot portion of the digest (commonly 10 cc) was pipetted into a 100-cc volumetric flask and diluted with 30 cc of water. Twenty-five cubic centimeters of a saturated solution of sodium carbonate was then added, followed immediately by 5 cc of Folin and Ciocalteu's (*3*) phenol reagent. After standing for one-half hour, the contents of the flasks were diluted to volume, and the color was compared with that developed by a tyrosine standard similarly prepared.

In tests not reported here it was found that this procedure gives a much higher result than can be accounted for by the sum of tyrosine and tryptophane when determined separately by the procedure cited. It was considered likely here that any changes occurring in this fraction would be due primarily to shifts in the amount of these amino acids. However, because of the possibility of other changes, the content of α-amino nitrogen in digests was determined also.

DETERMINATION OF α-AMINO NITROGEN

The method employed for determining α-amino nitrogen was a modification of the Sorensen formaldehyde titration. It was found that the comparator technique of Grünhut (*7*) and of Lüers (*9*) could be satisfactorily applied. The concentration of indicators and the volume of solutions at the time of comparison were carefully controlled. Titration was accomplished in two steps: (1) In aqueous solution to an end point corresponding to pH 8.5 (using phenolphthalein); and (2) from this end point to the same color after the addition of neutral formaldehyde. Titer 2 is a measure of the liberated amino nitrogen. It was found that the use of N/70 sodium hydroxide gave duplicate determinations of good agreement.

The preparation of neutral formaldehyde deserves special comment. It was observed that if 40-percent formaldehyde, adjusted to a faint pink with phenolphthalein, was diluted several times with water, a very noticeable increase in the indicator color resulted. When the neutralized formaldehyde was added to a sample containing a small amount of α-amino nitrogen (step 2) there resulted not the expected discharge of the pink color, but its intensification. This effect was noticed also by Mounfield (*10*), who eliminated the error through individual correction in each case by adding the sodium hydroxide equivalent of the apparent development of alkalinity. In the present investigation this error was eliminated by adjusting the formaldehyde to such a pH value as to give the desired shade of indicator upon dilution in the titration. Precautions were taken to insure the proper concentration of indicator.

RESULTS AND DISCUSSION

The results of the first several preliminary experiments were somewhat erratic but indicated, on the whole, a significant difference in the proteolytic activity of discoloring and normal tubers. Proteolytic activity was generally greater in the digests of discoloring tubers than in that of normal tubers. The average results of three of the more reliable tests are shown in figure 1, disclosing a substantially

greater activity in the abnormal tubers. Subsequent experiments were conducted over a longer period and the average results of three tests are shown in figure 2. These data show greater activity than preceding tests over the 6-day period because some of the earlier tests gave unusually low proteolytic activity. Differences in variety, age, size, and other qualifications of the tuber may account for the occasional variation of activity noted in different experiments. It was

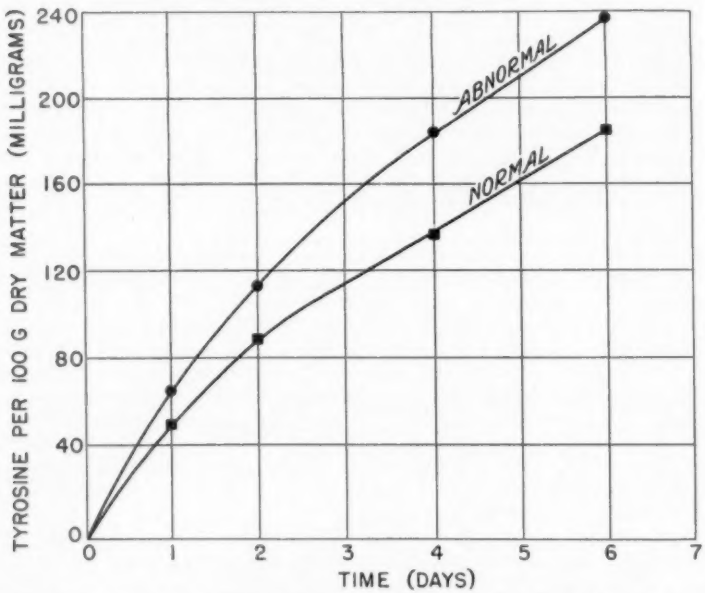

FIGURE 1.—Liberation of tyrosine during the autolysis of normal and of abnormal potato tissue in the first set of experiments.

found that the larger addition of water, 2 cc per gram of fresh tissue, entailed a slightly greater hydrolysis per gram of dry matter.

The data for α-amino nitrogen, as presented in figure 3, also show a significant increase of proteolysis in the discoloring tubers. However, the differences are not as great as in the case of the tyrosine equivalent. This relation is in agreement with the earlier evidence that tyrosine accumulates more than other amino acids in discoloring tubers and seems to be one of the first components released from protein (*1*, *5*). In spite of this difference in amino acid content of the tissue the isolated proteins appear to be identical in respect to the content of nitrogen, tyrosine, and tryptophane. The proteins were extracted with 5-percent solution of sodium chloride, reprecipitated several times with magnesium sulphate, and finally precipitated by alcohol. Tyrosine and tryptophane were determined by the method of Folin and Marenzi (*4*). Protein from normal potatoes contained 13.95 percent nitrogen, 5.37 percent tyrosine, and 0.52 percent tryptophane, while that from abnormal potatoes contained 13.90, 5.38, and 0.52 percent of the respective constituents. This content of tyrosine is

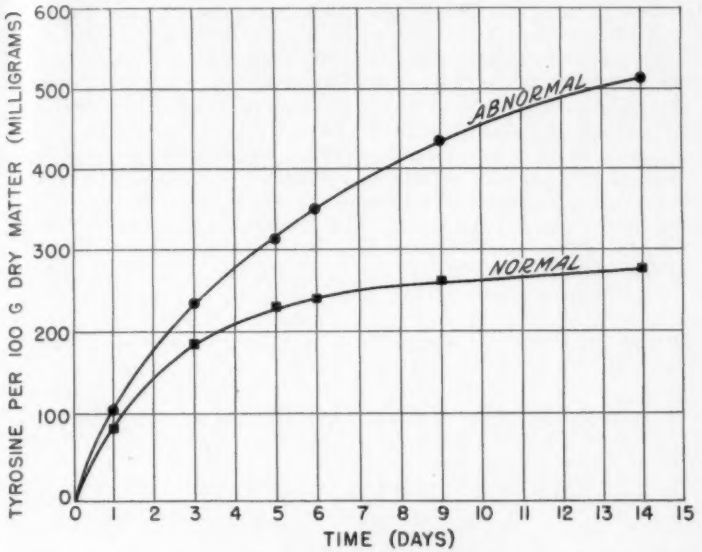

FIGURE 2.—Liberation of tyrosine during the autolysis of normal and of abnormal tissue in the second set of experiments.

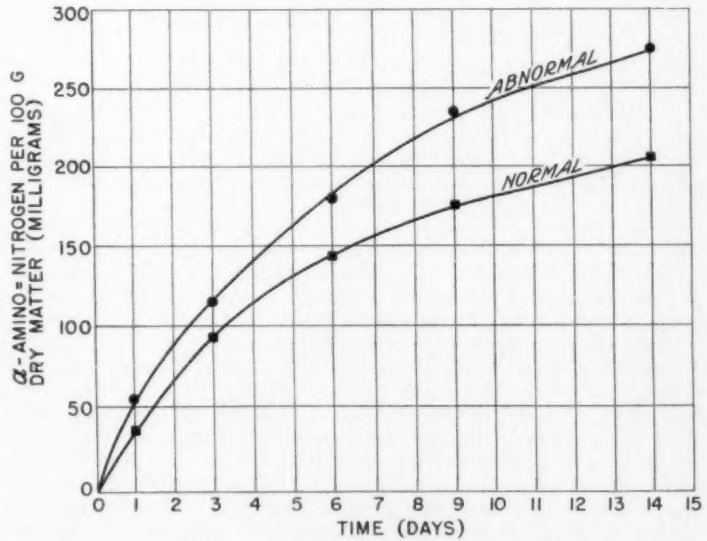

FIGURE 3.—Liberation of α-amino nitrogen during the autolysis of normal and of abnormal potato tissue.

higher than any previously reported for tuberin. Sjollema and Rinkes (*14*) reported 4.3 percent of this constituent. No report of the tryptophane content of tuberin has been found in the literature. Fürth and Lieben (*6*) applied alkaline hydrolysis to the tuber tissue and reported 0.2 percent tryptophane in the dry matter which they computed as equivalent to 3.3 percent in the protein. The constancy of tyrosine and tryptophane content in these protein preparations suggests that either this molecular complex is stabilized by precipitation in the abnormal tuber extracts or that polypeptides are involved in the greater susceptibility of such tubers to proteolysis. In the

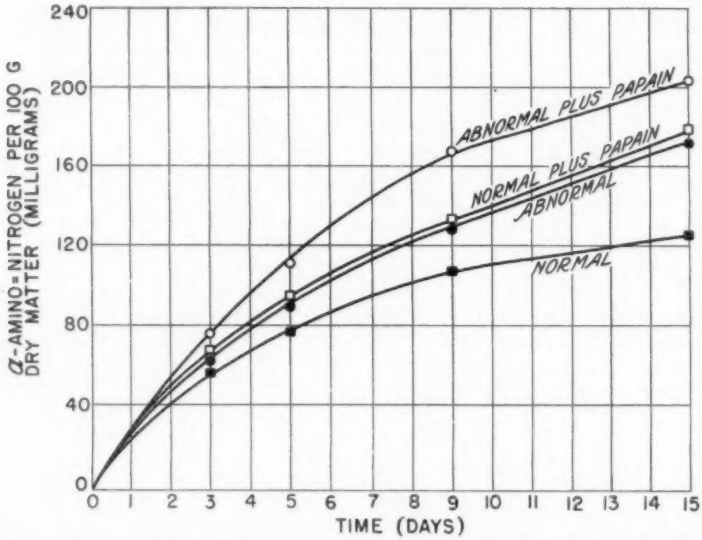

FIGURE 4.—Liberation of α-amino nitrogen in autolysates alone and in autolysates plus papain.

writers' earlier work (*15, p. 302*) the isolated proteins differed in ease of alkaline hydrolysis.

When the enzyme concentration was increased by the addition of papain an acceleration in the rate of hydrolysis resulted in each type of digest. The results of a single experiment are given in figure 4, but these data were substantiated by two tests which gave similar, though more irregular, curves. While the difference is generally rather small, the departures up to the twelfth day show somewhat greater acceleration by papain in the digests of abnormal tuber tissue. If there were inequality in enzyme activity of the two types of tissue, the addition of equal proportions of another enzyme should result in the suppression of differences in rates of autolysis. Relatively severe depletion of the substrate may have prevented more marked acceleration of proteolysis in abnormal tissue following the addition of enzyme. The results thus seem to be indicative of the presence of either easily hydrolyzed protein or lower polypeptides in discoloring pota-

toes. They do not preclude the possibility of difference in activity of the native enzymes.

A further matter of interest here is the relative positions at which the curves flatten. In the case of normal tubers, the curves of autolysis begin to level off about the seventh or eighth day (fig. 4). No such decline in rate of proteolysis was apparent in either the autolysates of abnormal tissue or the digests of normal tissue with papain. On the other hand, the curve for digest of abnormal tissue with papain (fig. 4) begins to flatten about the eleventh or twelfth day. Normally, one would expect the decline in rate of hydrolysis to occur first in the system in which hydrolysis is most rapid (abnormal tuber digests), as a more serious depletion of substrate would occur. It may be suggested that the more readily hydrolyzed substrate of discoloring potatoes could include polypeptides, a protein of low molecular weight, or an unstable form of a larger protein structure. When protein was added to the digests, proteolysis still proceeded at a somewhat greater rate in tissue from discoloring tubers, but the spread in activity was lessened. This effect offers limited evidence that the enzymes of the two tuber types are equally active on the added protein. In general, the results support earlier evidence (*15, p. 302*) of instability of the protein, as well as the concept of lower polypeptides, in the abnormal tubers.

It proved infeasible to add cyanide for the activation of protease because this ion interfered with the determination of tyrosine and tryptophane. The addition of sodium chloride for its possible solvent effect upon protein did not affect the rate of hydrolysis appreciably. An increase in the potassium concentration of digests of discoloring potatoes, making this factor essentially equal to that of normal ones by the addition of potassium chloride, did not decrease the rate of hydrolysis. The possibility is recognized that other sources of this metal might influence the rate of hydrolysis.

Consideration of the data for α-amino nitrogen shows that the differences were significant and not merely magnified by the method of expression as milligrams per 100 g of dry matter. Expressed as cubic centimeters of N/70 sodium hydroxide, the titration of 5 cc normal digest increased from 0.6 after 1 day to 3.6 after 14 days, while the discoloring digests showed increases over these levels of 50 and 36 percent, respectively. The addition of the doubled proportion of water (2 cc per gram) increased the release of amino nitrogen by about 50 percent but left the spread between samples undisturbed. Duplicate titrations generally agreed within 0.1 cc.

The liberation of more than 200 mg of α-amino nitrogen per 100 g of dry matter, as observed in the present work, represents a considerable hydrolysis of protein. This is evident from the fact that there is about 5 percent of true protein in the dry matter of potato tubers, which is equivalent to about 800 mg of total nitrogen in 100 g of dry matter.

During the course of this investigation, data were accumulated on the content of α-amino nitrogen and of tyrosine equivalent in the tuber extract as prepared. By tyrosine equivalent is meant the entire reacting capacity of the extract obtained, when sodium carbonate is added before the phenol reagent in the usual manner. Calculated on the basis of the total dry matter, normal tubers contained 0.35 percent of α-amino nitrogen and 0.43 percent of tyrosine equivalent. Discol-

oring tubers were 28.6 percent and 39.5 percent higher in content of these respective constituents. These differences are much greater than those reported previously (*15, p. 299*), but in the earlier examination the tubers had not been indexed for discoloration after cooking. Furthermore, a different type of extract was used here, namely, sap acidulated with trichloroacetic acid, whereas in the earlier work alcohol to give about 60 percent concentration was employed.

SUMMARY

Reasons are given for considering abnormal enzymic activity as a possible cause for the blackening of potatoes after cooking.

Procedures are described for the investigation of autolysis in potato tuber tissue, and modified methods for the determination of α-amino nitrogen and of a tyrosine equivalent are given.

Discoloring tubers show a considerable increase of the tyrosine equivalent during autolysis as compared with normal tubers. The latter release over 25 percent of their protein or peptide nitrogen as α-amino nitrogen under favorable conditions for autolysis, but there is an appreciable increase of this function in discoloring tubers.

Proteins prepared by salting out from the saps of normal and discoloring potatoes contained equal amounts of both tyrosine and tryptophane. The amounts of these constituents differ from values previously reported.

Earlier evidence of the presence of abnormal proportions of free amino acids and tyrosine in discoloring potatoes is confirmed. By using indexed tubers, an increase of nearly 30 percent in amino nitrogen and about 40 percent in tyrosine equivalent was found in discoloring potatoes as compared with normal ones.

Sodium chloride, used as a solvent for tuberin, did not increase the rate of proteolysis. Potassium chloride did not suppress the rate of proteolysis, thus indicating that a deficit of this form of potassium was not the direct cause of accumulation of amino acids.

Added proteins were hydrolyzed at essentially equal rates in tissue digests of the two types of tuber. From this it appears that increase of free amino nitrogen in discoloring tubers is not due primarily to abnormal activity of proteolytic enzymes.

Papain increased proteolysis somewhat more in the digest of discoloring potatoes. These results are interpreted as indicative of either decreased stability of proteins or increased proportions of lower polypeptides as causal factors in the increased free amino acid content of the abnormal tubers.

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MORPHOLOGY OF THE DIGESTIVE TRACT OF THE BLACKFLY (SIMULIUM NIGROPARVUM)[1]

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INTRODUCTION

Comparatively little information exists in the literature relative to the internal anatomy of the family Simuliidae. Hungerford (*6*)[2] has figured and described the internal anatomy of *Simulium vittatum* Zett., and Smart (*11*) in a recent paper has described the internal anatomy of *S. ornatum* Mg.

In recent years it has been found that certain species of blackflies transmit disease-producing organisms. Blacklock (*1*, *2*) has demonstrated that *simulium damnosum* Theob. is an intermediate host of a filarial worm *Onchocerca volvulus* Leuckart, which causes a disease of man. O'Roke (*10*) has shown that a blood protozoon of ducks, *Leucocytozoon anatis* Wickware, passes the sexual stage of its life cycle in *Simulium venustum* Say and that the organism is transmitted by the bite of the fly.

During the past 2 years the blackfly *Simulium nigroparvum* Twinn has been found feeding on turkeys in southwestern Virginia, and Johnson and Underhill (*8*) have shown that this fly is responsible for the transmission of a blood protozoon disease of turkeys. In view of the recent discovery that *S. nigroparvum* is an important vector of this blood protozoon of turkeys, the writer felt that a detailed study of the internal anatomy of the fly would be of service in studying the life history of the protozoon. Specimens of *S. nigroparvum* were readily accessible, and a detailed study was made of the internal morphology of this fly. Since only the females feed on turkeys and the males are unimportant as possible vectors, the digestive tract of the female only is described.

TECHNIQUE

Dissections were made on freshly killed specimens. For sectioning, the material was fixed in Bouin's fluid. All material was dehydrated with ethyl alcohol and cleared in terpineol. Hemalum and Delafield's haematoxylin were used for nuclear stains, and fast green and eosin for counter stains.

GROSS ANATOMY

The fore intestine (stomodaeum) is represented by the oesophagus and its diverticulum, the crop with its duct, and part of the cardiac valve. The mid intestine (mesenteron or ventriculus) is a simple tube, the anterior portion of which is narrow and the posterior portion

[1] Received for publication January 8, 1938; issued September 1938.

[2] Italic numbers in parentheses refer to Literature Cited, p. 447.

of which is expanded and is capable of considerable distention. The hind intestine (proctodaeum) comprises the distal intestine, the Malpighian tubules, the rectal pouch and glands, and the rectum proper.

MOUTH PARTS AND SUCKING APPARATUS

The mouth parts of several species of blood-sucking Nematocera have been figured and described by several workers, among whom may be mentioned Dimmock (*4*), Kellogg (*9*), Emery (*5*), Cameron (*3*), Jobling (*7*), and Smart (*11*). The mouth parts of *Simulium nigroparvum* are the piercing, sucking type and are quite similar to those described by Cameron (*3*) for *S. simile* Mal. and by Smart (*11*) for *S. ornatum*. Observations made on living females of *S. nigroparvum* have demonstrated that the mode of biting is quite similar to that of *Culicoides pulicaris* L. as described by Jobling (*7*).

The mouth parts and biting apparatus are shown in plate 1. In general character the mouth parts are short and broad. The labrum-epipharynx (pl. 1, *F*) is a broad triangular structure which is kept rigid by three rodlike sclerotizations. At the tip of the labrum-epipharynx are two sclerotized trifid structures.

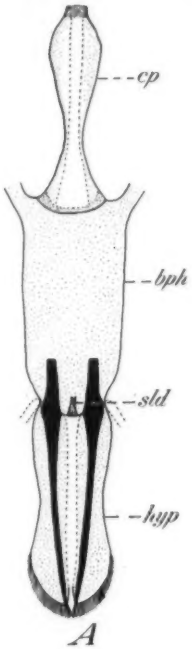
The hypopharynx (pl. 1, *A*) is a long narrow sclerotized rodlike structure the tip of which is armed with bristles. The salivary duct enters the proximal portion of the hypopharynx and is carried along the cephalic surface to the distal end.

The basipharynx is a flattened channel formed by the union of the hypopharynx and epipharynx. The labium (pl. 1, *B*) is short and broad and the theca appears to consist of two separate parts but actually they are fused at the base. The large, free labella curve round and forward, and when the insect is biting surround the other mouth parts. The proximal parts of the labella are rigidly sclerotized while the distal portions are soft and bear numerous setae on the surface.

The maxillae lie along the outer margins of the labrum and hypopharynx. They are spear-shaped structures, the anterior face of which is flattened and is armed with teeth around its margin (pl. 1, *E*). The maxillary palps consist of five segments and are covered with numerous setae. The third segment bears a depressed saclike are which is probably a sensory vesicle (p. 1, *C*).

The mandibles are short, thin, swordlike structures fitted for piercing, and are armed with teeth on their anterior margins (pl. 1, *D*). Extending up the blade of each mandible from the marginal teeth are prominent striations. In the center of each mandible is a clear, crystallike area. The mandibles are so shaped that, for the greater part, the blades overlap between the labrum and the hypopharynx.

According to Snodgrass (*12*) the sucking apparatus of Diptera is the cibarium of orthopteroid insects and therefore would be called the cibarial pump. Also, the functional mouth aperture leading into the pump is actually not a true mouth. The true mouth occurs at the opening into the stomodaeum at the inner end of the pump. The cibarial pump of *Simulium nigroparvum* is triangular in section and consists of one dorsal sclerotized plate and two ventrolateral sclerotized plates. The pump is operated by muscles inserted on the plates (pl. 2, *C*).



A

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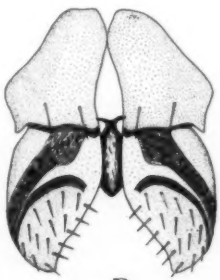

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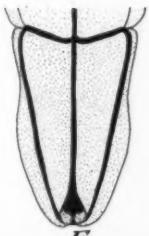

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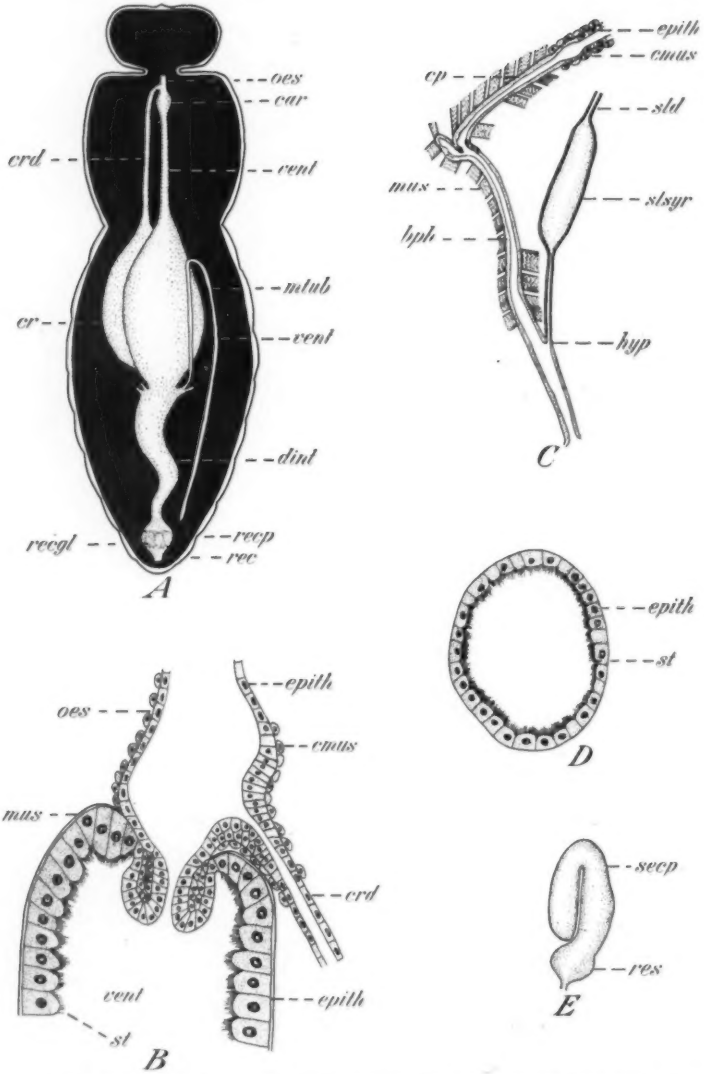
Mouth parts of *Simulium nigroparvum*: *A*, Hypopharynx, basipharynx, and cibarial pump; *B*, labium *C*, maxillary palp; *D*, mandible; *E*, maxilla; *F*, labrum epipharynx: *bph*, Basipharynx; *hyp*, hypopharynx *cp*, cibarial pump; *sld*, salivary duct.



Simulium nigroparvum: *A*, diagrammatic dorsal view showing the alimentary canal and Malpighian tubules; *B*, longitudinal section through cardiac valve; *C*, sagittal section through basipharynx and cibarial pump; *D*, cross section through anterior portion of ventriculus; *E*, salivary gland. *bph*, Basipharynx; *cmus*, circular muscle; *crd*, crop duct; *cr*, crop; *dint*, distal intestine; *epith*, epithelium; *hpy*, hypopharynx; *mtub*, malpighian tubule; *mus*, muscle; *cp*, cibarial pump; *oes*, oesophagus; *car*, cardia; *rec*, rectum; *recgl*, rectal gland; *recp*, rectal pouch; *res*, reservoir; *secp*, Secretory part; *sld*, salivary duct; *stsyr*, salivary syringe; *st*, striated margin of cell; *vent*, ventriculus.

SALIVARY GLANDS

The salivary glands, which are paired, lie in the anterior part of the thorax dorsad to the digestive tract. Each salivary gland is in the form of a U and is composed of two parts, viz, the anterior secretory part and the round saclike reservoir (pl. 2, *E*). A duct comes off from the reservoir of each gland. The two ducts from the salivary glands pass down, one on each side of the intestine, and unite beneath the oesophagus. The common duct leading from the point of junction expands to form the salivary syringe (pl. 2, *C*). From the salivary syringe the duct narrows and passes to the hypopharynx.

STOMODAEUM

The oesophagus, which is continuous with the cibarial pump, is a short, narrow tube that extends posteriorly between the head ganglia through the cervix, and joins the cardia of the ventriculus in the anterior part of the thorax. Within the thorax anterior to the cardia is the evaginated crop duct coming off the ventral side of the oesophagus and extending caudad beneath the ventriculus into the abdomen. Here it enlarges into an oblong thin-walled sac. The crop is capable of considerable distension and occupies most of the anterior part of the abdominal cavity of flies which have fed on sweetened water. However, no blood was found in the crop of flies which had fed on turkeys. The cardiac valve (pl. 2, *B*) marks the junction of the fore and mid intestine.

MESENTERON

The mid intestine, or ventriculus, is a straight tube of varying diameter. The anterior part is narrow and tubelike and extends along the median line of the body to the abdomen where it expands over the crop, the degree of expansion depending on the amount of blood the fly has consumed. The mid intestine is marked at its anterior end by the cardiac valve and at the posterior end by the pyloric valve. The posterior portion of the mid intestine narrows slightly where it joins the hind intestine.

PROCTODAEUM

The hind intestine is marked anteriorly by the pyloric valve and posteriorly by the rectum. At the point of junction of the mid and the hind intestine four Malpighian tubules arise, one pair on each side of the intestine. They are of considerable length and follow an irregular course through the abdominal cavity.

The anterior part of the hind intestine is a slightly tapering tube which may or may not be curved, depending on the development of the ovaries. This is known as the distal intestine. The posterior part of the hind intestine forms a flask-shaped sac called the rectal pouch, which contains six rectal glands. The rectal glands are arranged in a circle around the opening of the distal intestine and are visible through the thin walls of the rectal pouch.

The rectum is a short tube which extends from the rectal pouch to the anus.

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HISTOLOGY OF THE ALIMENTARY TRACT

MOUTH PARTS AND BITING APPARATUS

The mouth parts, being true appendages of the head, have the same structure as the body wall. The chitinous lining of the food channel is heavy and rigid. It is overlain by a very thin layer of epithelium; and bands of muscles extend from the body wall to the labrum-epipharynx and the labium-hypopharynx.

The walls of the basipharynx are heavy, chitinous structures overlain by a thin layer of epithelium. Muscle bands are attached to its anterior face.

SALIVARY GLANDS

The salivary glands are tubular and are composed of a single layer of cells whose bases rest on the basement membrane. The elongated secretory portion of each gland is composed of large cuboidal cells and the reservoir part is made up of small flattened cells (pl. 3, *D*). From the reservoir part of each gland a slender duct arises, which unites with that of the other gland to form the common salivary duct. A short distance from the point of junction, the duct expands to form the salivary syringe, whose walls are strengthened by riblike sclerotizations (pl. 2, *C*). The walls of the salivary duct are composed of a chitinous intima and a very thin layer of epithelium. No muscle layer was found on either the glands or the duct.

STOMODAEUM

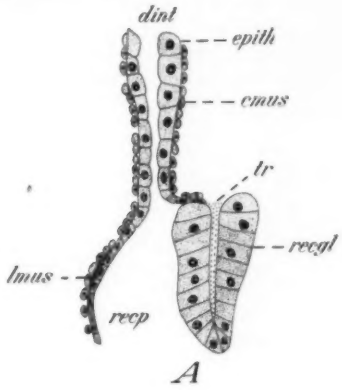
The oesophagus and its diverticulum, the crop, are true stomodeal structures and show a similar histological structure. The chitinous intima is so thin that it cannot be detected in most sections. Also, a thin layer of epithelium is found in all parts and is composed of flattened cells joined at their bases to a basement membrane. Covering the epithelium is a layer of circular muscle fibers. In the crop both the epithelial and the muscle layer are extremely thin.

The oesophagus extends well into the ventriculus and is represented by a single layer of epithelial cells. The inability to distinguish a chitinous intima makes the determination of the limits of the fore intestine difficult. It is also difficult to determine the ectodermal from the endodermal tissue in sections through the cardiac valve.

MESENTERON

The mid intestine, or ventriculus, differs from the fore and the hind intestine in histological structure. The inner and most conspicuous layer of the stomach is that of the epithelium, the bases of whose cells rest on the basement membrane (pl. 2, *D*). Following the basement membrane is a very thin layer of circular muscles and over these are scattered longitudinal muscles. The cells of the epithelium are columnar (pl. 3, *G*) and are sometimes thrown into irregular folds. The epithelium of the posterior part of the stomach is variable in thickness and, when the gut is distended with food, is only about one-third the thickness of the normal epithelium (pl. 3, *C*). The epithelial cells in all parts of the mid intestine show a striated inner margin.

The epithelium of the mid intestine functions in the secretion of digestive fluids. The secretion is merocrine inasmuch as the entire cell does not burst. Sections taken through the stomach of an unfed



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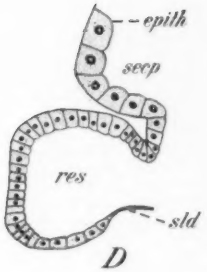

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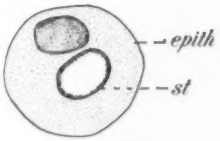

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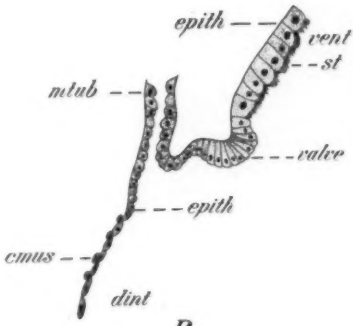

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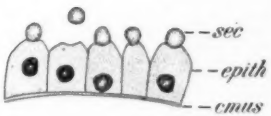

F

C

G

Simulium nigroparvum. *A*, Longitudinal section through a portion of the distal intestine and one rectal gland; *B*, longitudinal section through pyloric region; *C* longitudinal section through the ventriculus of an engorged fly showing reduced epithelium; *D*, longitudinal section through salivary gland; *E*, cross section through Malpighian tubule; *F*, longitudinal section through ventriculus of an unfed fly; *G*, longitudinal section through ventriculus showing cuboid epithelium. *cmus*, Circular muscle; *dint*, distal intestine; *epith*, epithelium; *lmus*, longitudinal muscle; *mtub*, malpighian tubule; *recgl*, rectal gland; *recp*, rectal pouch; *res*, reservoir; *sec*, secretion; *secp*, secretory part; *sld*, salivary duct; *st*, striated margin of cell; *tr*, tracheal branch; *vent*, ventriculus.

fly show a very active secretion taking place (pl. 3, *F*). The inner end of the cells bud off and there is no rupturing of the cell wall. During the process of secretion the epithelial cells do not show the striated border.

PROCTODAEUM

The hind intestine is marked anteriorly by the pyloric valve. This valve is formed by a ring of epithelial cells at the end of the mid intestine. These cells, unlike those found in the stomach, have no striated border. The lip of the valve encircles the intestine just anterior to the opening of the Malpighian tubules (pl. 3, *B*). The cells of the Malpighian tubules are of the characteristic cuboidal shape and are continuous with the epithelium of the hind intestine. A short distance from the point of attachment, the cells of the Malpighian tubules are narrow and elongated with conspicuous nuclei. Their inner margins are distinctly striated (pl. 3, *E*).

The epithelium of the distal intestine consists of flattened cells, which are raised into folds in the posterior part. Covering the epithelium is a layer of circular muscles which forms a continuous layer at the posterior end. As in the fore intestine, the intima in the distal intestine is too thin to be readily demonstrated.

The walls of the rectal pouch are extremely thin and the epithelium is greatly reduced except where it forms the rectal glands. Both muscle layers are very thin and the rectal glands are readily visible through the walls of the rectal pouch. The rectal glands (pl. 3, *A*) are composed of large cells with prominent nuclei. The lumen of each gland is occupied by a tracheal branch. The chitinous intima is very thin and not easily demonstrated in the rectal pouch.

The posterior end of the rectal pouch narrows abruptly to form the rectum. Here both muscle layers as well as the epithelium are well developed and the chitinous intima lining the rectum can easily be detected.

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SEASONAL VARIATIONS IN THE CARBOHYDRATE AND NITROGEN CONTENT OF ROOTS OF BEARING PECAN TREES [1]

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INTRODUCTION

In the pecan (*Hicoria pecan* (Marsh.) Britton), as in other perennial plants, the early growth of the tree in spring is determined largely by the food reserves that have accumulated during the previous growing season and are made available in spring. These food reserves play a part in developing resistance to cold during winter and are utilized for new shoot and root growth, initiation of pistillate flowers, production of catkins and pollen, and the initial development of the nut.

Previous analyses of roots of top-worked pecan trees [3] indicated that there was considerable storage of food reserves in the roots and that a heavy nut crop materially affected the seasonal variation of the stored constituents. Since the pecan generally bears only in alternate years, and in view of the importance of food reserves in the growth, development, and fruiting performance of the tree, it seemed desirable to obtain some information on the proportionate seasonal accumulation of these food reserves in the roots of bearing pecan trees and the effect of a crop on their accumulation.

REVIEW OF LITERATURE

LeClerc du Sablon (*12, v. 16*)[4] analyzed the roots, stems, and leaves of various deciduous trees, including the chestnut, pear, and peach, for sucrose, starch, hemicellulose, and moisture. In general, he found that the total carbohydrate reserves in the roots pass through a maximum in autumn at the time of leaf fall, diminish a little in winter, and diminish greatly during growth in the spring. After growth has subsided and assimilation is at its height, reserve carbohydrates increase and attain their maximum again in autumn. The carbohydrate content of the roots was higher than that of the stems and showed more variation, indicating that the roots are more important storage organs than the stems. The extensive variation of hemicellulose indicated that it may be an important reserve. The variation of sucrose was relatively slight, and LeClerc du Sablon concludes that sucrose is a less important reserve material than the form in which other carbohydrates become mobile and assimilable.

Later LeClerc du Sablon (*12, v. 18*) worked with several evergreen trees. In all cases he found that the total carbohydrates in the roots

[1] Received for publication December 28, 1937; issued September 1938.

[2] The writers wish to express their appreciation to L. D. Romberg for determining the dry matter and assisting in preserving samples during the progress of this investigation.

[3] SMITH, C. L., HAMILTON, J., THOR, C. J. B., and ROMBERG, L. D. EFFECTS OF VARYING DEGREES OF SEVERITY IN HEADING BACK LARGE PECAN TREES IN TOP-WORKING ON THEIR SUBSEQUENT ROOT COMPOSITION AND TOP DEVELOPMENT. [In manuscript.]

[4] Italic numbers in parentheses refer to Literature Cited, p. 459.

decreased during leaf formation and new shoot development and increased after this growth was completed, although the growth periods occurred at different times of the year. He also analyzed the stems, roots, and leaves of the evergreen oak and Japanese spindle tree for nitrogen and found that in the roots it decreased in spring during new leaf formation, passed through a minimum in summer, and increased toward winter. He concluded that in spring nitrogen moves from the roots and stems to the newly formed leaves and, when growth ceases, continues to be absorbed and is stored, thereby increasing in late summer and toward winter.

Butler et al. (*2*) analyzed different parts of the apple tree and found that the reserves were stored chiefly as starch and sucrose, the roots and branches containing relatively more starch than the trunk. Davis (*6*) found that the roots and trunks of nonbearing Sugar prune trees contained much more starch than those of bearing trees, and that the starch concentration in the roots was much greater than in the trunks. He states that it is reasonable to suppose that root growth is suppressed when the demand for carbohydrates above ground becomes great, and suggests that fruit trees, which have a heavy annual crop that remains on the trees most of the summer, may experience a shortage of carbohydrates for the roots. He also noted that starch was the most variable of the carbohydrates. Cameron (*3*), working with young orange trees, noted a minimum of starch in the roots in August, then a gradual increase to a maximum in early spring, and a gradual decrease to a minimum again in August. An inverse relation between starch and moisture was indicated. For the greater part of the year, the starch content in the roots was higher than in the leaves, branches, or trunk, and the greatest starch fluctuation occurred in the root bark. Murneek (*16*), investigating the carbohydrate storage of bearing apple trees, noted the unusually high starch and sugar content of the roots in autumn. He concluded that the underground parts of the apple tree serve as storage organs for carbohydrates, primarily starch. His data indicate that there is an increase in total sugars and a slight decrease in hemicellulose, starch, and total carbohydrates in the roots from early November to late December.

The importance of hemicellulose as a reserve material has been stressed by a number of investigators working with parts of the tree other than the roots. Schellenberg (*18*), in a summary of such investigations, concludes that hemicellulose serves as a reserve material and its deposition enables the plant to survive the long rest periods (winter or dry period) into which it is forced at times. Tottingham et al. (*21*) and Murneek (*14, 15*), working with apple spurs, noted extreme fluctuations of hemicellulose and emphasized its significance as an available form of carbohydrate in the spur. Its accretion in the fruit reached a maximum in midsummer and then decreased rapidly, thus indicating that hemicellulose must be a source of sugars for the flesh of the apple. Hooker (*11*) found an extensive variation in hemicellulose in apple spurs, which seems to indicate that it functions as a reserve material. Murneek and Logan (*17*), investigating the autumnal migration of nitrogen and carbohydrates in the apple tree, concluded that with the onset of cold weather starch and possibly other more complex carbohydrates are hydrolyzed simultaneously into sugars in all peripheral

regions of the tree. They state that modifications induced by weather often are of greater amplitude than possible seasonal trends.

Finch (*7*, *8*) concluded that for best filling of pecan nuts in Arizona the vegetative growth of trees must be checked during the early summer to provide conditions for carbohydrate storage during the summer months. He found that starch was formed in the current-season shoots in summer but disappeared completely during the nut-filling period. This emphasizes the importance of an abundant supply of carbohydrates for conversion to fats in nut filling.

Finch and Van Horn (*9*) found that starch storage in bearing pecan shoots reached a maximum shortly before nut filling began, and then decreased; whereas a comparable decrease did not occur in vegetative nonfruiting shoots. In the latter starch continued to accumulate, reaching a maximum in early fall. These authors conclude that the starch from the fruiting shoots is used in nut filling. Thor and Smith (*20*) have shown that most of the oil in pecan kernels is formed within a period of 4 to 6 weeks and that most of the carbohydrate material for its formation must be brought in from outside the nut during this period.

The maintenance of healthy foliage on the fruit tree has been emphasized by Heinicke and Hoffman (*10*) and other workers. Sitton (*19*) ringed bearing shoots of the pecan in early August after the shells of the nuts had begun to harden, but before any considerable filling had taken place, and noted that the best filling was obtained in nuts on shoots that bore the largest number of leaves per nut. Crane et al. (*5*) conclude that the almost universal tendency of pecan trees to bear more or less irregular crops from year to year cannot be called either biennial or alternate bearing and that without doubt the controlling factors are the nutritional condition and previous performance of the tree. Their data indicate that nut thinning may be expected to increase the annual yield per tree by maintaining a more nearly optimum nutritional condition in the tree at all times. Smith et al.,[3] in studies on top-working pecan trees, note the value of the leaf area in the functioning of the tree. Chemical analyses of the roots showed that with severe heading the balance of the carbohydrate reserves in the tree is disturbed to a greater degree than in trees with less severe topping. As the top is rebuilt the amount of these materials and their functions become more nearly like those of the normal tree. Crane and Hardy (*4*) have shown that cultural treatments, such as pruning or applications of nitrogen, which stimulate shoot growth with subsequent increase in leaf area, are of great importance in determining the ultimate size of pecan nuts and the degree to which they are filled.

MATERIALS AND METHODS

Since the pecan is frequently an alternate bearer, the progress of this investigation was facilitated by the use of trees in an alternate bearing condition during "off" and "on" years. During the first season of the experiment there was no nut crop; in the second season the crop was large; in the third season the crop was very small.

Seventy-five healthy seedling trees about 45 years old, growing in a cultivated river-bottom field about 15 miles from Austin, Tex.,

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were selected and divided into 15 comparable groups of 5 trees each. The average diameter of the trees was about 25 inches. Composite root samples for chemical analysis were taken from these groups in rotation in time intervals ranging from 2 weeks to approximately 1 month. Owing to the death of the farm owner and subsequent litigation of ownership of the property, it was necessary to discontinue the investigations from October 1935 to August 1936. A summarized description of the condition of the trees during the course of the 3 years is given in the last column of table 1 (p. 457).

SAMPLING METHODS

Samples of lateral roots with diameters ranging from three-eighths to one-half inch were taken, brought immediately to the laboratory, chopped fine, and mixed thoroughly. Samples to be dried were killed by placing them in an oven at 100° C. for 1 to 2 hours and were then dried to constant weight in a vacuum oven. Two 50-g portions were transferred immediately to 500-ml Erlenmeyer flasks with sufficient boiling 95-percent alcohol to give a final alcohol concentration of 80 percent by weight. The flasks were then closed with short-stemmed funnels and heated 30 minutes on a water bath kept at 90° to 95°. The flasks were stoppered while still hot and stored in the dark until the samples were used for carbohydrate analyses.

ANALYTICAL METHODS

Whenever the nature of the material permitted, the official methods of the Association of Official Agricultural Chemists (*1*) were employed.

Dry Matter

Samples that had been weighed into aluminum cans and placed in ovens at 100° C. for 1 to 2 hours were transferred to a vacuum oven and dried to constant weight at a temperature of 80° and a pressure of less than 1 mm of mercury. The resulting dry material was ground in a drug mill or food chopper and stored in glass bottles, and was redried under the same conditions before it was used in further analyses.

Total Organic Nitrogen

No analyses were made for nitrate or nitrite nitrogen since previous analyses had shown no appreciable amounts of these forms of nitrogen in pecan roots. The procedure for determination of total organic nitrogen followed closely the Kjeldahl-Gunning-Arnold method except for the use of copper wire as the digestion catalyst. In general 2-g portions of the dried material were used. The preliminary drying of the sample at 100° C. for 1 to 2 hours has been shown by Link and Schulz (*13*) to have little or no effect on the total nitrogen content of plant tissues.

Sugars

The alcohol-preserved samples were transferred to large Soxhlet extractors with 80-percent alcohol and extracted for 22 to 24 hours on a water bath kept at 95° to 99° C. The alcohol extract was distilled almost to dryness in vacuo at temperatures not above 60°. The residue was taken up with water, treated with 25 cc of basic

lead acetate to clear the solution, and made up to a volume of 250 ml. The lead acetate solution used for clearing was made up of 500 g of basic lead acetate in 1 liter of solution. The cleared filtrate was deleaded with anhydrous disodium phosphate.

The Munson-Walker method was used for the determination of sugars in aliquots of the lead-free filtrate. Reduced copper was determined by the volumetric permanganate method. For total sugars, inversion was accomplished by means of acid at room temperature as directed in official methods (*1, p. 187,* [*23c*]). Reducing-sugar values were calculated as invert sugar and nonreducing sugars as sucrose.

Starch by Diastase

The alcohol-insoluble residues were transferred to tared aluminum cans, dried in an air oven at 100° C., and weighed. After being ground in a drug mill the material was stored in aluminum cans in a desiccator until ready for analysis.

The official method for determination of starch in feeding stuffs by diastase with subsequent acid hydrolysis (*1, p. 120,* [*23*]) was used with the exception that a 0.5-percent solution of taka diastase was substituted for the malt extract. Preliminary extractions with cold water were eliminated since sugars had already been extracted with alcohol.

Starch by Direct Acid Hydrolysis

Alcohol-insoluble residues were used as in the diastase method. The official methods (*1, p. 119,* [*21*]) were used. As before, no preliminary extractions with cold water were necessary.

Those carbohydrates that are not hydrolyzed by the taka-diastase method for starch determination, but are hydrolyzed in the direct acid hydrolysis method, are designated as hemicelluloses. The hemicellulose values were obtained by subtracting the starch by taka-diastase values from the corresponding values for starch by direct acid hydrolysis.

EXPERIMENTAL DATA

Since no investigations were made on the variability of the constituents in comparable samples obtained at the same time, no particular emphasis can be attached to the analytical data for any individual sample, and the results must be viewed from the standpoint of trends. The analytical data are presented in figure 1.

DRY MATTER

The percentage of dry matter in the roots was higher in the summer and fall of 1934 than at any other time during the experiment. This was probably due to a deficiency of soil moisture during an extreme drought which lasted until November. After the drought was broken by rains the percentage of dry matter in the roots decreased rapidly until about January 1935. Between January and September there was only a slight decrease in dry matter, but from the middle of September to the latter part of October there was a considerable decrease. From August 1936 to February 1937 the dry matter fluctuated considerably between sampling dates, but showed no consistent increase or decrease.

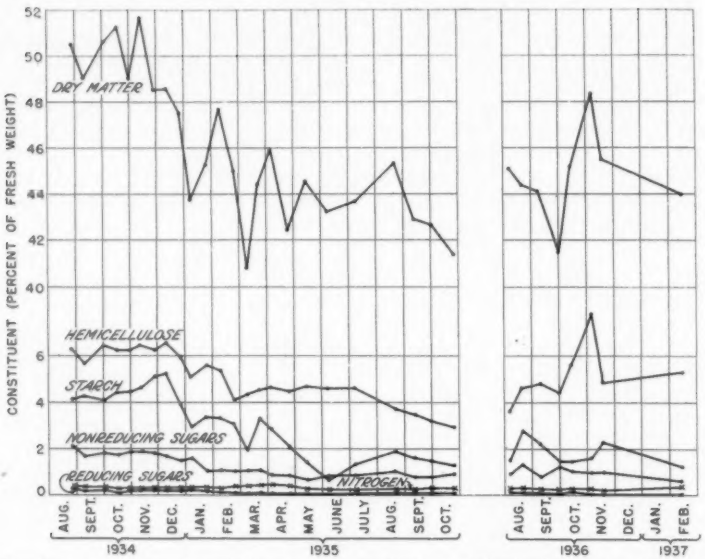

FIGURE 1.—Seasonal variation in dry matter, carbohydrates, and nitrogen in roots of pecan trees.

The following tabulation shows the monthly rainfall record at Austin, Tex., from January 1, 1934, to February 20, 1937, inclusive:

Year and month:	Rainfall (inches)
1934	
January	7.43
February	5.01
March	2.37
April	3.31
May	.90
June	.32
July	.39
August	.24
September	2.61
October	.32
November	3.40
December	4.59
1935	
January	.95
February	2.96
March	1.20
April	2.08
May	9.21
June	9.71
July	1.44
1935—Continued	
Year and month—Continued.	Rainfall (inches)
August	.24
September	8.79
October	1.65
November	.85
December	2.84
1936	
January	0.39
February	1.70
March	1.52
April	.66
May	8.15
June	3.30
July	9.25
August	2.90
September	5.22
October	2.63
November	2.30
December	1.88
1937	
January	2.43
February	.12

This record indicates that the roots had an abundant moisture supply throughout the experiment except in the summer and fall of 1934. The rainfall was low in July and August of 1935 but there was sufficient rain in June to carry the trees through this period without a moisture deficiency.

SUGARS

The concentration of reducing sugars was very low throughout the experiment and showed no consistent seasonal variations. The nonreducing sugars were present in appreciable quantities and fluctuated as starch did below but to a lesser extent. The highest concentration occurred in the late summer and fall of 1934. There was a decrease during the winter and spring, due in part to the decrease in dry matter. From May 13 to July 6 there was an appreciable increase in the nonreducing sugars, after which their concentration remained practically constant until October 25. On August 6, 1936, the concentration of these sugars was at the same level as in October 1935, but it increased considerably during August and then remained practically constant until November 18. Between the latter date and February 16, 1937, there was a material decrease similar to that in the winter of 1934–35.

STARCH

The concentration of starch increased slightly from August to December 1934, decreased rapidly to January 1935, and then decreased more slowly to June 7. There was no nut crop on the trees in 1934, and the increase in starch content of the roots was probably due to the photosynthetic activity of the leaves and translocation of carbohydrates to the roots where they were stored as starch. This seems to be further indicated by the comparatively high concentration of nonreducing sugars present in the roots during this period. From December 1934, after most of the leaves had fallen or had become inactive from frost, there was a rapid decrease in concentration of starch to January 1935. A decrease in carbohydrates in roots after leaf fall has been noted by other investigators (*12*, *16*) and indicates a translocation of these substances to the aerial portions of the tree. From January 7 to March 22 the starch showed only slight changes except for one low value on March 8. After March 22 the starch decreased rapidly, reaching a minimum on June 7. During the latter period the initiation of growth took place, shoots and leaves developed, the trees blossomed, and the development of the nuts was begun. All these processes create heavy demands upon the tree for carbohydrates. The leaves during most of this period are immature and small, and their total photosynthetic activity is very low and therefore inadequate to supply the necessary carbohydrates for the rapid growth and fruiting activities. It is interesting to note that the concentration of the hemicellulose remained practically constant during this period notwithstanding the fact that from December 10 to February 23 it decreased in about the same proportion as starch.

From June 7 to August 20, 1935, there was a considerable increase in the starch. During this period the shucks and shells of the nuts were being developed, but other vegetative growth was probably nearly completed for the season. The leaves were probably at their most efficient stage for photosynthesis and therefore were able to produce a greater amount of carbohydrates than was required for tree activities, some of the surplus being stored in the roots as starch.

From August 20 to October 25, 1935, the starch content of the roots decreased slightly. About September 1 the period of nut filling begins and the demand for carbohydrates during this period has been shown to be very great (*20*). This is especially true when the trees are carrying a large crop of nuts, as was the case here. Both starch and

hemicellulose concentrations decreased during the nut-filling period from September 1 to the latter part of October. A part of this decrease was due to a decrease in the dry matter of the roots, but calculations show that both starch and hemicellulose decreased in greater proportion than the dry matter. Thus it seems probable that some of the hemicellulose as well as the starch was utilized in the nut-filling process. No doubt the photosynthetic activity of the leaves was high during the nut-filling period, but with a heavy crop of nuts on the trees the demand for carbohydrates for oil formation was greater than could be supplied by photosynthesis, and some of the reserves in the tree were used.

After the 9-month interval from October 25, 1935, to August 6, 1936, during which no samples were taken, the starch concentration showed a slight increase, which may be accounted for by the increase in the percentage of dry matter during this period. However, the starch concentration was still not so high in proportion to the dry matter as it was in 1934. The starch fluctuated considerably during the fall of 1936 but showed no consistent increase. On February 16, 1937, when the last samples were taken, the starch was low, amounting to 1.26 percent of the fresh weight. The nut crop was small in 1936 and therefore made much less demand for carbohydrates for nut filling than the large crop of 1935, but the starch content of the roots on the average was only slightly higher than for the corresponding period in 1935. Conditions were very favorable for vegetative growth in 1936, however, and the starch was probably utilized for this purpose.

HEMICELLULOSE

The percentage of hemicellulose was at its highest level in the summer and fall of 1934, when the dry matter was at its maximum. Its fluctuation was almost proportional to that of the dry matter from August 1934 to January 1935. From January to October, however, the percentage of hemicellulose shows a greater proportional decrease than the percentage of dry matter. This indicates that there was an actual decrease in the amount of hemicellulose in the roots and that only a part of the variation in its concentration was due to changes in the concentration of dry matter. The trees bloomed heavily in the spring of 1935 and matured a large crop of nuts. Therefore it is possible that some of the hemicellulose was utilized in the development and filling of the nuts. From October 1935 to August 1936 the percentage of hemicellulose had increased a little but not in proportion to the increase in dry matter. From the latter part of 1936 to February 1937 the hemicellulose increased gradually, but at the end of this period it was still somewhat lower in proportion to dry matter than in the fall of 1934.

NITROGEN

The percentage of total organic nitrogen decreased slightly from August 27, 1934, to January 7, 1935 (fig. 1 and table 1). This decrease may be accounted for by the decrease in the concentration of dry matter. There was a gradual increase in the nitrogen concentration from January 7 to April 5, probably because the roots absorbed more nitrogen than was translocated away. There was little growth activity in the tree during this period, while at the same time soil moisture and probably other factors were optimum for the absorption of nitrogen.

TABLE 1.—*Seasonal variation in total organic nitrogen in pecan roots and growth and fruiting condition of trees*

Sample No.	Sampling date	Total organic nitrogen	Growth and fruiting conditions
	1934	*Percent* [1]	
1	Aug. 27	0.38	Foliage on all trees apparently fully mature and in healthy condition. No apparent extension of shoot growth. No nut crop on trees.
2	Sept. 10	.39	
3	Oct. 2	.26	
4	Oct. 16	.31	
5	Oct. 30	.29	
6	Nov. 12	.34	About 3 percent of leaves have dropped.
7	Nov. 27	.30	Trees have lost about 40 percent of foliage and owing to frost leaves are turning yellow and dropping rapidly.
8	Dec. 10	.32	
9	Dec. 26	.33	Defoliation complete.
	1935		
10	Jan. 7	.32	Trees dormant.
11	Jan. 23	.39	
13	Feb. 8	.35	
15	Feb. 23	.40	
17	Mar. 8	.40	
19	Mar. 22	.45	Buds bursting into growth on a few trees.
21	Apr. 5	.52	Most trees well into growth and a few almost in full leaf. Heavy pistillate bloom.
22	Apr. 24	.42	Pollen shedding very active.
23	May 13	.34	Shoot growth well under way and active.
24	June 7	.35	Shoot growth nearly complete. Large crop of nuts set.
25	July 6	.27	Shoot growth complete and foliage mature.
27	Aug. 20	.32	Nuts in "watery" stage.
29	Sept. 12	.33	Nuts in "dough" stage.
31	Sept. 30	.37	Nuts beginning to ripen.
32	Oct. 25	.33	Nuts mature and dropping from trees. Foliage dropping rapidly.
	1936		
35	Aug. 6	.33	Foliage full grown and in good condition. Extension of shoot growth apparently continuing through most of this period. Nut crop very small.
36	Aug. 20	.31	
37	Sept. 9	.34	
38	Oct. 1	.34	
39	Oct. 13	.32	Nuts ripening.
40	Nov. 6	.37	Leaves beginning to fall. Nuts fully mature and dropped.
41	Nov. 18	.32	Trees almost completely defoliated.
	1937		
42	Feb. 16	.40	Trees dormant.

[1] Percentage based on fresh weight.

From April 15 to May 13, 1935, the nitrogen decreased to about its 1934 level, after which it remained practically constant for the remainder of the experiment, except that it had increased appreciably between November 18, 1936, and February 16, 1937.

The decrease in nitrogen in April and the first part of May may be ascribed to the intensive vegetative activity of the trees at this time. The initiation of shoot growth and the development of blossoms took place during this period and consequently created a heavy demand for nitrogen. Conditions were no doubt favorable for the absorption of nitrogen at this time, but the demand was probably greater than could be supplied by root absorption, and some of the reserve nitrogen from the tissues was used in the growth functions. After the intensive vegetative activity of the tree was over there was a large crop of nuts to be developed and filled; therefore the nitrogen did not increase again during that season. In the fall of 1936 the nut crop was light, but conditions for vegetative growth of the trees were more favorable than in 1935 and the growth extended later into the season, so that all the nitrogen absorbed was required in growth processes. After the growth season was over the nitrogen increased appreciably during the winter. In 1934 the low level of nitrogen in the roots may have been due to the extremely dry weather, which created unfavorable conditions for absorption of nitrogen by the roots.

DISCUSSION

Starch was the most variable constituent determined in the roots of pecan trees. The data indicate that it increases from early summer to a maximum in late fall and decreases to the summer minimum, but the levels of the maxima and minima seem to be determined by the growth and fruiting conditions during the time. Both growth and fruiting in the pecan are exhaustive processes, as has been found in other trees by Murneek (*14*), Davis (*6*), and other investigators. The starch in pecan roots was built up to a high maximum concentration in the fall of 1934, when there was no nut crop, and the vegetative growth was at a standstill owing to a severe drought. With the intensive spring growth and blossoming of the trees in 1935 the starch decreased to a very low level and did not increase during the summer and fall because of the great demand for carbohydrates in filling the large crop of nuts. There was also a decrease in the hemicellulose content of the roots during the nut-filling period. This indicates that both starch and hemicellulose may function as carbohydrate reserves and are used in the filling of the nuts or in other processes when the demand is sufficiently great.

It is not known how low a level the starch concentration reached in the fall of 1935, since no samples could be taken after October 25 until August 6 of the following year. However, on the latter date the starch concentration was still relatively low and did not increase materially during the autumn notwithstanding the fact that the nut crop was very small. Conditions were very favorable for vegetative growth throughout the season, and the failure of the starch to increase in the autumn can be explained only on the assumption that tree growth continued throughout the fall, thereby utilizing the carbohydrates, in this process and in the filling of the small crop of nuts, as fast as they were synthesized.

In the spring of 1936 and in the spring of 1937 the bloom was light and the starch in the autumn preceding each of these seasons was at a low concentration, whereas in the fall of 1934 the starch reached a high concentration which was followed by a very heavy bloom in the spring of 1935. Whether the lack of reserve carbohydrates, as indicated by starch, was the cause of the small bloom in the 2 later years is not known, but this same condition has been found in other trees (*6*, *11*) and seems to be either directly or indirectly concerned with fruiting. The fact that the pecan seldom, if ever, bears two large crops in successive years and that fruiting is a very exhaustive process indicates that the amount of storage carbohydrates, especially starch, is a very important factor in fruiting whether the effect be direct or indirect. Davis (*6*) found that the starch content of roots of nonbearing Sugar prune trees was much higher than that of roots of bearing trees, and concludes that storage carbohydrates may be essential for fruit-bud differentiation or that other factors may cause fruit-bud differentiation because of their relationships to the storage carbohydrates.

The nonreducing sugars were present in appreciable quantities and fluctuated with the starch, although to a lesser extent. The wide variations in concentration of these sugars may indicate that they function as storage reserves. However, the fact that they fluctuated so nearly with the starch may indicate that they are labile forms and that their concentration is largely dependent upon that of the starch.

Although the concentration of nitrogen was relatively low throughout the experiment, the data indicate that the amount of nitrogen in pecan roots is governed partly by conditions favorable to absorption and partly by the nitrogen requirements for tree growth and fruiting. In the latter part of 1934 the nitrogen concentration was low, but it increased from January to April in 1935. The summer and fall of 1934 were extremely dry and the soil moisture was doubtless a limiting factor in the absorption of nitrogen. From January until April 1935, however, soil moisture was adequate for absorption, and the trees were dormant and therefore required very little nitrogen. With the initiation of spring growth and blossom development the demand for nitrogen was great and probably could not be met by root absorption; hence the nitrogen decreased in the roots. The heavy nut crop of 1935 made a considerable demand for nitrogen in nut filling, and the nitrogen did not increase during that time. However, from November 1936 to February 1937, when the trees were dormant, the nitrogen again increased materially as in the first part of 1935.

SUMMARY

Composite samples of lateral roots of pecan trees of bearing age were collected at intervals during an "off" and an "on" year and analyzed for the principal carbohydrates and total organic nitrogen.

The starch concentration tended to reach a maximum in late fall, after which there was a winter decrease and then a further decrease in spring to a minimum in early summer. The amount of starch in the roots for any period was apparently dependent upon the relative growth rate of the tree, age and condition of foliage, and the size and stage of development of the nut crop.

Starch was the most variable of the constituents determined, and its concentration appeared to have a marked influence on fruiting.

The rapid disappearance of starch during the spring growth and blossoming period and the decrease of starch and hemicellulose during the nut-filling period show that both growth and fruiting in the pecan are exhaustive processes.

Reducing sugars were low and showed no consistent seasonal variations, thus indicating they are labile forms of carbohydrates. The nonreducing sugars, however, were present in appreciable quantities and varied with the starch although to a lesser extent. These may serve as storage carbohydrates or may be labile forms whose concentration depends largely on that of starch.

The total nitrogen content in the roots was low at all times, but it decreased during rapid spring growth and increased during winter when the trees were dormant and soil conditions were favorable for nitrogen absorption. Nitrogen concentration was largely independent of dry-matter content.

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APPLICABILITY OF NUTRIENT-SOLUTION PURIFICATION TO THE STUDY OF TRACE-ELEMENT REQUIREMENTS OF RHIZOBIUM AND AZOTOBACTER [1]

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INTRODUCTION

Though almost 20 years have elapsed since the introduction by the writer of the method of nutrient-solution purification for studying trace-element requirements of plants, no application of this procedure to studies with bacteria has as yet been made. For reasons readily apparent, it has long seemed desirable to the writer to obtain data affording a direct comparison of the effects of this technique on bacteria and on *Aspergillus niger* Van Tiegh. (*14*).[3] A direct comparison involves the use of the same equipment, chemicals, and technique by the same investigator, so far as possible. The organisms selected for this comparison were *Rhizobium trifolii* Dangeard and *Azotobacter chroococcum* Beij.

The procedures employed necessitated, as usual, the ascertainment of the minimum quantities of the usual ash constituents required for maximum growth, together with the identification and study of the necessary trace elements. Again, it was found essential to devote some attention to the accessory growth substance required by rhizobium in order to limit the extent to which any mineral impurity it might contain would alter or vitiate the results on mineral nutrition.

Though these experiments are, in a manner, incomplete, it should be recalled that their primary objective was a study of the applicability of the method of nutrient-solution purification to bacterial studies. It was considered undesirable, therefore, to go more deeply into the study of these organisms than was necessary for a general comparison with aspergillus. To have done so, even for the purpose of obtaining improved results with the trace elements, would have necessitated changes in equipment, to provide adequate aeration for azotobacter, besides considerable additional work on purification of the accessory growth bodies required by rhizobium.

REVIEW OF LITERATURE

No attempt will be made to present a general summary of the literature, in view of the many excellent reviews that are available. That of Wilson (*18*) on rhizobium and of Burk (*4*) on azotobacter are among the most recent.

[1] Received for publication June 4, 1938; issued September 1938.

[2] The writer expresses his appreciation of the help extended him by various investigators in the U. S. Department of Agriculture. L. A. Burkey, of the Bureau of Dairy Industry, made the microscopic bacterial counts mentioned in this paper, and Daniel Ready, of the Division of Soil Microbiology, Bureau of Plant Industry, the nitrogen determinations. Acknowledgment is further made of cultures and helpful information received from L. T. Leonard and N. R. Smith, also of the latter Division.

[3] Italic numbers in parentheses refer to Literature Cited, p. 476.

Rhizobium and azotobacter are known to require, besides a source of energy, nitrogen, potassium, phosphorus, magnesium, sulphur, iron, calcium, and molybdenum. Various claims also have been made regarding the necessity for other elements, including copper, zinc, manganese, tungsten, silicon, vanadium, and strontium. Furthermore, complete agreement has not as yet been reached regarding the essentiality of an accessory growth factor for rhizobium (*17*). On the other hand, data have begun to accumulate that would indicate that, like the vitamin requirements of animals, the accessory growth-substance requirements of all bacteria and fungi are limited to a circumscribed number of organic compounds the need for which is general and not specific to a single organism and the functions of which in plants are quite analogous to those of vitamins in animals.

METHODS

Rhizobium trifolii Dangeard (E. B. Fred No. 205) and *Azotobacter chroococcum* Beij. (N. R. Smith No. 7) were grown for 4 days at 28° C. in 50-cc portions of a sucrose nutrient solution in 125-cc pyrex Erlenmeyer flasks. Stock cultures of *R. trifolii* were maintained on an agar medium containing sucrose, bactopeptone, malt, and yeast extract, while those of *A. chroococcum* were grown on Ashby agar. Three drops of a freshly prepared bacterial suspension was used to inoculate each flask. Sucrose containing not over 0.0025 percent of ash, reagent chemicals, and water redistilled in pyrex glass were used in the preparation of the nutrient solutions. The flasks were sterilized at 15 pounds' pressure for 30 minutes.

Purification (*14*) with magnesium carbonate was accomplished by heating at 100° C. in the Arnold sterilizer for 20 minutes (10 minutes being allowed for prewarming); and with calcium carbonate, by heating for the same length of time at 15 pounds' pressure in the autoclave. The quantities of carbonate differed in different experiments, the details of which are given later. The nutrient solutions were treated before addition of the trace elements and the organic compounds to be tested. Filtration was through a Jena glass filter of No. 4 porosity.

Each experiment tabulated was the final test of a series performed to determine the optimum concentrations of the individual constituents. The experiments tabulated are typical tests, comprising only 5 to 10 percent of those performed.

Cultures were usually prepared in duplicate and were quite uniform as a rule. The references to the differences in yields of the controls in different experiments do not apply to the data of the same experiment. Reproducibility and consistency of relative results in repeated trials are emphasized rather than the absolute results of an individual experiment.

A Bausch & Lomb nephelometer, provided with 50-mm cups, was used to measure growth as turbidity. Use of a fluctuating turbidity standard, namely, control cultures, proved quite unsatisfactory, since the controls themselves remained unknowns, but this defect was remedied by improvising a turbidity standard consisting of a rod of turbid acrylate condensate. The turbidity of all cultures was measured with the cup at 10 mm. Each millimeter of the turbidity standard was assumed to be equivalent to approximately 10,000,000

rhizobium cells per cubic centimeter or approximately 2,000,000 azotobacter cells per cubic centimeter. The estimates were based on readings of 49.2 mm with a rhizobium suspension of 500,000,000 bacteria per cubic centimeter and 11.3 mm with an azotobacter suspension of 22,320,000 bacteria per cubic centimeter, on the assumption of an exact proportionality between growth and turbidity. A freshly prepared solution containing 25 mg of magnesium sulphate ($MgSO_4.7H_2O$) plus 50 mg of potassium phosphate (K_3PO_4) per 100 cc gave a reading of 25.2 mm with the solution cup set at 10.0 mm. Acidities were determined with a Leeds and Northrup quinhydrone electrode.

The values in table 1 are of interest in view of the scarcity of such data and the increasing use of the nephelometer for measuring bacterial growth as turbidity. They are the results of several different experiments.

Though these observations are limited in both number and range covered, they would seem to indicate a change in proportionality between bacterial count and turbidity depending upon the extent of growth. The average value of the ratio between bacterial count and turbidity is 2,009,000 for readings between 2.0 and 11.3 mm. An increased precision should be readily attainable through the use of a more carefully calibrated, uniform turbidity standard, manipulation to obtain numerical readings of greater magnitude, and mechanical means to form a uniform suspension of bacteria. Also, the use of a colloid to stabilize the suspension may be advisable. The rapidity with which readings may be made would warrant further investigation of the possibilities of the nephelometer.

TABLE 1.—*Comparison of growth measurements of azotobacter as determined by turbidity readings and by microscopic bacterial counts*

Turbidity (millimeters)	Bacteria	Ratio of number of bacteria to turbidity	Turbidity (millimeters)	Bacteria	Ratio of number of bacteria to turbidity	Turbidity (millimeters)	Bacteria	Ratio of number of bacteria to turbidity
	Millions per cubic centimeter	*Millions per millimeter*		*Millions per cubic centimeter*	*Millions per millimeter*		*Millions per cubic centimeter*	*Millions per millimeter*
0.5	5.336	10.672	2.5 (−Fe)	2.828	1.131	3.8	7.360	1.937
1.0	8.372	8.372	2.5 (−Mo)	4.692	1.877	4.5	13.104	2.912
1.7	7.304	4.297	2.8	8.677	3.099	11.3	22.320	1.975
2.0	2.376	1.188	3.7	6.120	1.654			

EXPERIMENTAL RESULTS WITH RHIZOBIUM

After considerable effort to grow rhizobium in solutions free from organic matter other than sucrose, further attempts were abandoned and Difco bactopeptone was adopted as a source of growth factor. It was found that yields of only 1 to 2 mm (2 to 4 million bacteria per cubic centimeter) could be obtained without the addition of organic material containing an accessory growth factor, whereas yields corresponding to 45 mm or more could be obtained by its addition. Repeated tests with salts of 77 of the chemical elements at different concentrations gave no indication to substantiate the belief that the poor growth without growth factor was due to any single inorganic deficiency. The quantity of bactopeptone to be employed was set at

50 mg per liter, i. e., 5 parts per 100,000. This is a somewhat suboptimal quantity, but it was adopted because of the turbidity characteristics of the cultures at the time of harvest after 4 days of growth.

The results given in experiment 5 (table 2) are typical of those obtained with an unpurified solution containing 50 mg of bactopeptone per liter, except that the yield of the control varied somewhat from experiment to experiment. The nonaddition of iron or of molybdenum to the nutrient solution did not result in a decrease in growth, while a slight decrease took place through withholding manganese or calcium. It is interesting to compare these findings with those of experiment 7 (table 2), in which a partially purified bactopeptone preparation in absolute alcohol was used. The use of this preparation, of which more will be said later, evidently had decreased the content of mineral impurities in the bactopeptone since a decrease in the percentage of maximum yield followed the omission of any one of the trace elements. Purification of the nutrient solution with magnesium carbonate seemed effective to the same degree, as reference to experiment 6 shows. The best results, however, were obtained by combining purification of the nutrient solution with the use of purified bactopeptone, as indicated in experiment 8.

Many additional experiments, other than those tabulated, led to the same conclusions: Omission of iron, manganese, molybdenum, or calcium from the nutrient solution gave decreased growth of rhizobium. No evidence, however, could be obtained for the essentiality of any of the other elements tested.

Experiment 3 (table 2) shows the effect of substituting sodium nitrate ($NaNO_3$) for ammonium nitrate (NH_4NO_3) in the nutrient solution, and experiment 2 the result of the omission of inorganic nitrogen. The solution in the latter experiment contained 13 mg of nitrogen per liter, owing to the addition of bactopeptone. Under these conditions, no fixation of nitrogen occurred in the control.

TABLE 2.—*Effects of trace elements on the growth of Rhizobium trifolii in different solutions for 4 days at 28° C.*

Trace element omitted	Effects with solution [1] used in—																							
	Experiment 1 (sucrose, 20.00; NH_4NO_3, 0.15; K_2HPO_4, 0.15; $MgSO_4.7H_2O$, 0.09; bactopeptone, 0.05; $4MgCO_3.Mg(OH)_2.5H_2O$, 0.40 g per liter, filtered hot)			Experiment 2 (sucrose,[2] 10.00; K_2HPO_4, 0.15; $MgSO_4.7H_2O$, 0.09; bactopeptone, 0.05; $4MgCO_3.Mg(OH)_2.5H_2O$, 0.50 g per liter, filtered hot)			Experiment 3 (sucrose, 10.00; $NaNO_3$, 0.32; K_2HPO_4, 0.15; $MgSO_4.7H_2O$, 0.09; bactopeptone, 0.05; $4MgCO_3.Mg(OH)_2.5H_2O$, 0.50 g per liter filtered hot)			Experiment 4 (sucrose, 5.00; NH_4NO_3, 0.15; K_2HPO_4, 0.15; $MgSO_4.7H_2O$, 0.09; bactopeptone, 0.05; $4MgCO_3.Mg(OH)_2.5H_2O$, 0.50 g per liter, filtered hot)			Experiment 5 (sucrose, 5.00; NH_4NO_3, 0.25; K_2HPO_4, 0.08; $MgSO_4.7H_2O$, 0.04; bactopeptone, 0.05 g per liter)			Experiment 6 (sucrose, 3.00; NH_4NO_3, 0.15; K_2HPO_4, 0.15; $MgSO_4.7H_2O$, 0.09; bactopeptone, 0.05; $4MgCO_3.Mg(OH)_2.5H_2O$, 0.50 g per liter, filtered hot)			Experiment 7 (sucrose, 3.00; NH_4NO_3, 0.25; K_2HPO_4, 0.08; $MgSO_4.7H_2O$, 0.04 g per liter; bactopeptone, 10 cc)			Experiment 8 (sucrose, 3.00; NH_4NO_3, 0.20; K_2HPO_4, 0.15; $MgSO_4.7H_2O$, 0.07; $4MgCO_3.Mg(OH)_2.5H_2O$, 0.50 g per liter; bactopeptone, 10 cc. Filtered hot)		
	Yield [3]	Proportion of maximum yield	Acidity at harvest	Yield [3]	Proportion of maximum yield	Acidity at harvest	Yield [3]	Proportion of maximum yield	Acidity at harvest	Yield [3]	Proportion of maximum yield	Acidity at harvest	Yield [3]	Proportion of maximum yield	Acidity at harvest	Yield [3]	Proportion of maximum yield	Acidity at harvest	Yield [3]	Proportion of maximum yield	Acidity at harvest	Yield [3]	Proportion of maximum yield	Acidity at harvest
	Mm	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*
Fe	9.1	26.2	7.37	7.6	73.8	7.27	24.3	66.4	6.80	12.0	43.0	7.38	18.2	148.0	5.03	10.2	52.6	7.17	11.7	80.1	4.89	7.4	31.1	7.05
Mn							34.3	93.7	6.66	25.8	92.5	7.04	11.3	91.9	4.92	17.2	88.7	7.15	8.2	56.2	4.58	14.4	60.5	7.03
Mo	35.8	102.9	6.74	9.9	96.1	7.02	30.8	94.2	6.59	25.5	91.4	6.95	12.9	104.9	4.78	19.2	99.0	7.06	12.3	84.3	4.55	20.8	87.4	6.97
Ca	31.9	91.7	6.59	8.4	81.6	7.02	29.1	79.5	6.64	21.7	77.8	7.07	10.9	88.6	4.78	15.7	80.9	7.18	12.4	84.9	4.55	12.9	54.2	7.07
None	34.8	100.0	6.54	10.3	100.0	6.81	36.6	100.0	6.39	27.9	100.0	6.88	12.3	100.0	5.18	19.4	100.0	6.64	14.6	100.0	4.84	23.8	100.0	6.82
Maximum [4]	39.4			12.7			40.2			28.6			19.2			21.8			15.2			29.4		
pH [5]			8.00			7.75			7.30			7.80			6.79			8.26			7.17			8.33

[1] Iron, manganese, molybdenum, and calcium were added to the extent of 0.06, 0.01, 0.01, and 0.30 mg per liter, respectively. All constituents of experiment 1 were in minimum concentration for maximum growth with 20-percent sucrose.
[2] This solution contained 13 mg N per liter initially and at harvest.
[3] The extent of growth was estimated by the turbidity of the culture. The values give the depths of the fixed turbidity standard required to match a 10-mm depth of the culture.
[4] Maximum individual yield obtained in the experiment.
[5] Initial acidity of the nutrient solution.

A comparison of the effectiveness of some organic substances and of their extracts in aiding the growth of rhizobium is given in table 3, since these results influenced the choice of bactopeptone as a source of growth factor. The results are stated as percentage increase in yield at a concentration of 1.0 mg per liter. Bactopeptone and yeast extract were most effective and to about the same degree. The difference in effectivity of these nitrogen-rich sources indicated that organic nitrogen as such was not the factor underlying these variations. Attempts to obtain the effective substance in purer form by simple extraction with solvents were unsuccessful. Nor was sublimation of bactopeptone at 147° C. and 5- to 10-mm pressure more successful. Though considerable material sublimed, its effectiveness was lower (3-percent increase at 1 mg per liter) than the original substance. Sublimation of a bactopeptone preparation obtained by precipitation with lead, adsorption with charcoal, and extraction with ammoniacal acetone seemed more efficacious, since a growth increase of 39.2 percent with approximately 1.0 mg per liter of substance was obtained in one trial.

Miscellaneous additional tests were made in the attempt to obtain a purer product. With 1.0 g of bactopeptone dissolved in 100 cc of

TABLE 3.—*Influence of organic substances on the growth of Rhizobium trifolii for 4 days at 28° C. in a nutrient solution purified with magnesium carbonate*

Organic substance added		Growth measured nephelometrically	Computed [1] increase in yield for 1 mg substance per liter
Material and method of production [2]	Quantity per liter		
	Milligrams	*Millimeters*	*Percent*
Control		1.2	
Bactopeptone, Difco	50.0	44.3	72.0
Extracted cold with 95-percent alcohol	13.0	14.9	87.7
Extracted cold with absolute alcohol	6.0	4.3	43.3
Extracted cold with acetone	.8	1.7	52.5
Extracted hot with 95-percent alcohol	23.6	18.9	62.3
Extracted hot with acetone	5.4	4.8	55.6
Nutrose, Pfanstiehl [3]	50.0	4.3	5.2
Extracted cold with 95-percent alcohol	3.6	3.4	41.7
Extracted cold with absolute alcohol	3.2	2.1	56.2
Extracted cold with acetone	2.2	1.6	60.0
Peptone, Witte	50.0	3.5	5.8
Yeast extract, Difco	50.0	53.9	88.0
Malt extract, Difco	50.0	23.5	37.2

[1] The assumption of exact proportionality is probably true only for the lower values (*10*), nor are corrections made for ash content.

[2] 1 gram of the solid was shaken with 100 cc of liquid and the clear supernatant solution tested. Hot extraction was with a Soxhlet extractor, the extract from 10 g being adjusted to 100 cc.

[3] In a previous experiment with an unpurified solution the yield of the control was equivalent to 1.3 mm; with 50 mg bactopeptone per liter it was 20.9 mm; and with 50 mg nutrose per liter it was 30.4 mm.

0.2 N sulphuric acid (H_2SO_4), it was found that growth factor was not destroyed by continued heating on the water bath. Neither was it precipitated by an excess of barium carbonate nor by phosphotungstic acid. The bactopeptone control, at 50 mg per liter, gave a growth value of 34.8 mm; the unheated control gave 35.2 mm; while that subjected to heating gave 34.2 mm. With barium carbonate ($BaCO_3$) the precipitated material gave a growth increase of 10.3 percent as compared to an increase of 56.1 percent in the unprecipitated material. With phosphotungstic acid, the growth increase in the

precipitated material was 9.6 percent and that in the filtrate 40.5 percent.

Treatment of a neutral solution of bactopeptone with basic lead acetate followed by deleading with potassium bicarbonate ($HKCO_3$) did not decrease the growth-factor content, as also reported by Clark, (7). If the solution was then treated with Merck's "Medicinal Charcoal, extracted with acid," the factor was adsorbed. Elution of the adsorbed factor was accomplished with ammoniacal acetone (1+9) or ammoniacal absolute alcohol (1+9). The latter gave a deep brownish-red solution and was the method by which the bactopeptone preparation used in some of the experiments of table 2 was prepared. This preparation could be freed of color with little loss in activity by diluting the neutral solution with water and extracting with amylic alcohol. The increase in effectiveness of the organic material by these methods was slight. The acetone extraction material, for example, gave a growth increase of 137 percent at the 1.0-mg level, as compared to an increase of 72 percent with the original substance.

Nutrose treated by the same procedures gave an increase in yield of 66 percent at the 1.0 mg per liter level, which was about equal to that obtained by simple extraction with absolute alcohol or acetone. Mild hydrolysis of nutrose did not lead to an increase in effectiveness.

Finally, the compounds in the following list were tested for their ability to bring about growth increases, but unsuccessfully. Lecithin (from eggs) sometimes brought about slight increases, the others practically none at all. Occasionally it was thought that one of these compounds was effective, but a careful check failed to verify this interpretation.

Glycerol
Raffinose
Trehalose
Maltose
Galactose
d-Mannose
d-Mannitol
Sodium glycerophosphate
Dextrose
Levulose
i-Inositol
Lactose
Malic acid
Asparagine
Malonic acid
Taurine
Cholesterol
Creatine
β-Carotene
Choline hydrochloride
β-Alanine
Tannic acid
β-Indolylpropionic acid
Glucosamine hydrochloride
Vitamin B_1
Vitamin C
Lactoflavine
Sodium magnesium chlorophyllin
Sodium iron chlorophyllin
Adenine
Uracil
Guanine
Hydroxylamine hydrochloride
Hydrazine hydrochloride
l-Tyrosine
dl-Valine
α-Alanine, racemic
d-Arginine
l-Aspartic acid
l-Cystine
d-Glutamic acid
Glycine
l-Histidine dihydrochloride
l-Hydroxyproline
l-Leucine
dl-Isoleucine
d-Lysine dihydrochloride
dl-Methionine
α-Phenylalanine
β-Phenylalanine
l-Proline
l-Tryptophane
Glycogen
dl-Serine
dl-Norleucine
Uric acid
Sodium nucleinate
Xanthine
Pyruvic acid
Glycolic acid
Thiourea
Allantoin
Lecithin (egg)

One interesting point had been encountered in these experiments on the purification of the accessory factor required by rhizobium; namely, that nutrose, when added to an unpurified nutrient solution, was as effective as bactopeptone in bringing about increased growth; whereas, when added to a solution purified with magnesium carbon-

ate [4] it was totally ineffective. The uninoculated control with the purified solution containing 50 mg of nutrose per liter usually had a value of 1 to 2 mm, while the inoculated control with nutrose had a value of 3 to 4 mm. The control without nutrose gave no growth. Evidently the magnesium carbonate treatment removed from the nutrient solution some factor, essential for growth of rhizobium, in which nutrose is deficient. This deficiency could not be supplied by subsequent addition of nitrogen, phosphorus, etc. Repeated tests with nutrose and salts of the chemical elements at concentrations of 50 to 500 parts per billion failed to identify the deficiency. The substances listed on page 465 were then tested in combination with nutrose and proved uniformly ineffective, with the exception of lecithin, which was the last one tried. One milligram of lecithin per liter in a purified solution containing 50 mg of nutrose per liter increased the growth of rhizobium from 2.7 mm to 39.4 mm, after correction for the turbidity caused by the nutrose. The need for the joint presence of nutrose and lecithin indicated that the presence of two accessories is required by rhizobium.

The possibility that the milligram of lecithin per liter added to the solution in conjunction with nutrose served as a means of replenishing a deficiency of nitrogen, phosphorus, etc., is quite remote. The quantity involved is too small. Nutrose itself contains nitrogen, phosphorus, and many inorganic impurities. Moreover, other organic compounds containing nitrogen and phosphorus could not overcome the deficiency. Lastly, and most important, is the fact that none of the solutions of experiments tabulated in this paper was deficient in phosphate, as ascertained by repeated tests.

The almost complete removal from the nutrient solution of the trace of an accessory factor, presumably introduced with the sucrose, by magnesium carbonate was duplicated with bactopeptone and barium carbonate ($BaCO_3$). To 2.5 mg of bactopeptone, dissolved in 5 cc of 0.2 N sulphuric acid, an excess of barium carbonate was added; the residue, after filtration, was extracted with ammoniacal absolute alcohol (1+9); and the solvent was evaporated. The uninoculated control in one experiment gave a value of 1.8 mm for the turbidity due to nutrose; the filtrate from the barium carbonate residue gave 2.2 mm; and the residue from the ammoniacal alcohol gave 21.0 mm. This procedure, therefore, was effective at a concentration of 0.5 mg bactopeptone per cubic centimeter in removing one or both accessories almost completely (about 98 percent) from the solution, but ineffective at a concentration of 10 mg of bactopeptone per cubic centimeter. No tests were performed to confirm the necessity for using an acid (or sulphate ion ?), instead of a neutral solution, for the precipitation. It should be noted in this connection that purification of the nutrient solution with magnesium carbonate removed the impurity of the sucrose almost completely, whereas the quantity removed when 50 mg of bactopeptone per liter was present made little perceptible difference in growth. Apparently barium carbonate was more effective than magnesium carbonate, if it is assumed that the presence of other constituents is immaterial.

[4] The composition of the purified nutrient solution was as follows: Redistilled water, 1,000 cc; sucrose, 20.0 g; NH_4NO_3, 0.30 g; K_2HPO_4, 0.12 g; $MgSO_4.7H_2O$, 0.06 g; and $4MgCO_3.Mg(OH)_2.5H_2O$, 0.10 g, filtered hot. To the clear solution was added nutrose, iron, molybdenum, and calcium, in quantities of 50.0, 0.05, 0.01, and 0.30 mg per liter, respectively. Its acidity was pH 7.5 to 7.8.

Tests finally were made with some of the growth accessories identified as necessary for certain micro-organisms. These were i-inositol, required by yeasts; pimelic and nicotinic acids, by the diphtheria bacillus (*12*); and vitamin B_1 by *Phycomyces* and other organisms (*6, 13*). *Staphylococcus* has also been found by Knight (*9*) to require nicotinic acid and vitamin B_1. Nutrose and lecithin, too, were included. They were tested singly and in pairs at 1 to 2 mg per liter but proved uniformly ineffective, except nutrose plus lecithin at 50 and 1 mg per liter, respectively.

As a consequence of the unsuccessful search for an accessory growth factor in relatively pure form the use of bactopeptone was adopted as standard. The use of alcoholic solutions of bactopeptone was found desirable to prevent bacterial decomposition and to decrease the ash content. Some decomposition of the accessory growth bodies seems to occur in alcoholic solution, however.

Little has appeared in the literature (*16*) on the necessity for a careful check of the various claims for the existence of growth factors, as well as of the presence of trace elements in the organic materials used to culture micro-organisms. The following tabulation should, therefore, prove of value as a measure of the extent to which organic substances, some of which are in constant use for the preparation of culture media, are contaminated with inorganic materials.

Contaminants found spectroscopically[1] *in some of the materials used in growth studies with rhizobium*

Material:	Elements identified spectroscopically[2]
Bactopeptone, Difco	Na, Ca, Mg, K, Al, Sr, Fe, P, Mn, Cu, Pb, Si, Ba(?), Li.
Peptone, Witte	Na*, Ca*, Mg*, Sr*, Fe*, Si*, B, Mn, K, Cu, Al, Sn, Pb, V(?), Ba.
Malt extract, Difco	Ca*, Mg*, Si, Cu, Mn, Fe, Al, P, K, Na, Ba, Sn, Pb, V(?), Ti(?), Sr.
Yeast extract, Difco	Fe*, Ca*, Mg*, Sr*, Na*, K*, Ba, Mn, Cu, Al, Pb, V(?), Si.
i-Inositol, Pfanstiehl	Ca*, Mg*, Fe*, Pb, Cu, Si, Al, V(?), Mn.
Cholesterol, Pfanstiehl	Cu, Mg(?).
Sodium magnesium chlorophyllin.	Ca*, Fe*, Sr, Cu, Pb, Al, V, Sn(?), Na, Mg, Si, Cd, Pt, Zn, Mn.
Casein, Pfanstiehl	P, Mg, Cu, Pb, Ag, Na, Zn(?).

[1] These data were obtained by B. C. Brunstetter, associate biochemist, Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, with a Bausch & Lomb large-size quartz spectograph and carbon arc.

[2] Strong traces are indicated by asterisks (*); doubtful, by question marks.

EXPERIMENTAL RESULTS WITH AZOTOBACTER

Cultural work with azotobacter also was handicapped by the inability to duplicate growth values for the controls in successive experiments despite care in the preparation of the nutrient solution, including the use of stock solutions of the inorganic constituents. The results with trace elements shown in table 4 have been duplicated frequently, however, and there can be little doubt, therefore, of their reliability. Azotobacter, like rhizobium, gave decreased yields when traces of iron, manganese, molybdenum, or calcium were omitted from nutrient solutions with both fixed and free nitrogen. This was evident even in the unpurified solution (experiment 1), and was plainer, at least for iron and calcium, with the purified solutions of experiments 2 and 3 (table 4).

TABLE 4.—*Effects of trace elements on growth of Azotobacter chroococcum in different solutions for 4 days at 28° C.*

Trace element omitted	Effect with solution [1] used in—																							
	Experiment 1 (sucrose, 20.00; K_2PO_4, 0.18; $MgSO_4.7H_2O$, 0.09 g per liter)			Experiment 2 (sucrose, 20.00; K_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.15; $4MgCO_3.Mg(OH)_2.5H_2O$, 0.10 g per liter, filtered hot)			Experiment 3 (sucrose, 20.00; K_2PO_4, 0.18; $MgSO_4.7H_2O$, 0.09; $CaCO_3$, 0.30 g per liter, filtered hot)			Experiment 4 (sucrose, 5.00, K_2PO_4, 0.18; $MgSO_4.7H_2O$, 0.09; NaCl, 0.20 g per liter)			Experiment 5 (sucrose, 5.00; $NaNO_3$, 0.18; K_2PO_4, 0.18; $MgSO_4.7H_2O$, 0.09; NaCl, 0.20 g per liter)			Experiment 6 (sucrose, 5.00; K_2PO_4, 0.09; $MgSO_4.7H_2O$, 0.09; $NaHCO_3$, 0.10 g per liter)			Experiment 7 (sucrose, 3.00; $NaNO_3$, 0.20; K_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.20; NaCl, 0.20; $4MgCO_3.Mg(OH)_2.5H_2O$, 0.30 g per liter, filtered hot)			Experiment 8 (sucrose, 3.00; $NaNO_3$, 0.20; K_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.20; NaCl, 0.20; $CaCO_3$, 1.00 g per liter, filtered hot)		
	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest
	Mm	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*
Fe	5.1	67.1	6.85	1.7	58.6	6.87	0.3	18.8	7.06	4.7	75.8	7.02	4.5	40.0	7.02	2.0	38.5	7.25	2.5	36.2	6.97	2.8	28.0	6.93
Mn	7.4	97.4	6.84	3.4	117.2	6.84	2.0	125.0	6.85	6.5	104.8	6.86	9.5	84.1	7.12	4.8	92.3	7.21	6.2	90.0	7.05	6.0	60.0	7.03
Mo	4.8	63.2	6.94	2.0	69.0	6.87	1.5	93.8	6.87	6.3	101.6	6.87	10.0	88.5	7.03	3.9	75.0	7.16	8.3	123.0	7.07	6.8	68.0	7.04
Ca	5.7	75.0	6.94	.5	17.2	7.26	.6	37.5	7.19	0	0	7.49	9.3	82.3	7.09	0	0	7.60	0	0	7.37	0	0	7.13
None	7.6	100.0	6.72	2.9	100.0	6.81	1.6	100.0	6.86	6.2	100.0	6.77	11.3	100.0	6.96	5.2	100.0	7.15	6.9	100.0	6.77	10.0	100.0	6.99
Maximum [3]	7.6			4.6			3.0			6.9			13.6			6.0			8.4			10.0		
pH [4]			7.94			8.07			8.32			8.06			8.09			7.22			7.55			7.47

[1] Iron, manganese, molybdenum, and calcium were added to the extent of 0.06, 0.03, 0.02, and 0.30 mg per liter, respectively. All constituents were in minimum concentration for maximum growth with 20-percent sucrose.

[2] The extent of growth was estimated by the turbidity of the culture. The values give the depths of the fixed turbidity standard required to match a 10-mm depth of the culture.

[3] Maximum individual yield obtained in the experiment.

[4] Initial acidity of the nutrient solution.

The growth with fixed nitrogen in experiment 5 (table 4) was almost double that obtained with free nitrogen. Moreover, the presence of fixed nitrogen as sodium nitrate ($NaNO_3$) did not eliminate the need for any of the trace elements required for growth with atmospheric nitrogen. Attention is also directed to the effect of sodium ion in experiments 4 to 8 (table 4), on the omission of calcium. The reason for the addition of sodium chloride was that it had been found capable, apparently, of causing increased growth, though this increase later proved to be fallacious. Other tests, moreover, had shown that though potassium phosphate (K_3PO_4) was present in optimum amount it could be halved in quantity without diminishing growth, if the deficit was replaced by potassium or sodium bicarbonate ($HKCO_3$ or $HNaCO_3$). Experiments 7 and 8 (table 4) illustrate the effects of purification with magnesium and calcium carbonate on the removal of trace elements in the presence of fixed nitrogen.

Aeration, it was assumed, was the limiting factor for growth with azotobacter in the preceding experiments. The use of 25 cc, instead of 50 cc, of nutrient solution in the 125-cc flasks, increased the growth of the control in experiment 7 (table 4) from 6.9 mm to 22.0 mm, and in experiment 8 from 10.0 mm to 27.8 mm. It is plainly evident, therefore, that none of the constituents of these solutions were deficient in quantity at the lower aeration level.

The effects of a somewhat higher but still insufficient aeration level are shown in table 5, in which 25 cc of culture solution was used in 200-cc flasks instead of 50 cc in 125-cc flasks as in the preceding experiments. Increased aeration brought about very marked increases in growth and slight increases in the concentrations of mineral constituents required. To judge from the fragmentary data available, optimum aeration for this strain would require the use of 1-liter Erlenmeyer flasks with not over 25 cc of nutrient solution, or a depth of liquid of about 3 mm. This is the depth employed by Kostytschew et al. (*11*), who obtained nitrogen fixation of about 25 mg of nitrogen per gram of sugar used. Here again the increase in growth with improved aeration demonstrated the presence of nutrient constituents in ample quantity for the level used.

The possibility of the complete substitution of calcium by strontium (*5*, *8*) was tested in a solution without fixed nitrogen, identical with that of experiment 6 (table 4). It had an initial reaction of pH 7.54 and growth in the control was lower than usual, being only 1.3 mm or about 2,600,000 bacterial cells per cubic centimeter (p. 463). Iron, manganese, molybdenum, and calcium were added, as usual, in concentrations of 0.06, 0.03, 0.02, and 0.30 mg per liter respectively. Omission of calcium resulted in a complete cessation of growth. The addition of strontium as chloride, in steps of 0.05 mg per liter up to a maximum concentration of 0.50 mg per liter, proved incapable of aiding growth in any degree in the calcium-free solution. The strontium salt contained 0.0013 percent of calcium, being practically spectroscopically pure. In other experiments in which only partial removal of calcium was accomplished, strontium, however, could be substituted for the deficiency, at least partially. A duplicate of experiment 1 (table 4) gave a yield of 5.5 mm and percentages of maximum yield of 58.2, 100.0, 103.6, and 25.5, respectively, for iron, manganese, molybdenum, and calcium. The initial reaction was

pH 8.16, and the reaction at time of harvest ranged from pH 6.74 for the control to pH 6.91 for minus calcium. Addition of strontium to the minus-calcium culture in equivalent quantity increased the yield to 72.7 percent of maximum, or 27.3 percent less than that of the control with calcium. Other tests gave about 82 and 70 percent replacement (controls, 7.6 and 5.7 mm). Reagent strontium chloride seemed equivalent to calcium chloride.

TABLE 5.—*Effects of trace elements on growth of Azotobacter chroococcum in different solutions for 4 days at 28° C. when 25 cc of nutrient solution was used in 200-cc flasks*

Trace element omitted	Effect with solutions [1] used in—											
	Experiment 1 (sucrose, 10.0; K_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.09 g per liter)			Experiment 2 (sucrose 10.0; K_2PO_4, 0.09; $MgSO_4.7H_2O$, 0.09; $HNaCO_3$, 0.10 g per liter)			Experiment 3 (sucrose, 10.0; K_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.20; $CaCO_3$, 0.50 g per liter, filtered hot)			Experiment 4 (sucrose, 10.0; $NaNO_3$, 0.20; K_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.20; $CaCO_3$, 0.50 g per liter, filtered hot)		
	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest	Yield [2]	Proportion of maximum yield	Acidity at harvest
	Mm	Pct.	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*	*Mm*	*Pct.*	*pH*
Fe	3.8	47.5	7.01	6.5	48.2	7.01	1.2	17.7	6.84	8.8	59.5	6.80
Mn	7.8	97.5	6.82	12.5	92.6	6.77	7.9	116.2	6.55	16.1	108.8	6.92
Mo	6.4	80.0	6.86	9.5	70.4	6.87	9.0	132.4	6.51	20.0	135.1	6.89
Ca	5.5	68.8	6.95	7.7	57.0	6.88	1.7	25.0	6.79	8.5	57.4	6.92
None	8.0	100.0	6.75	13.5	100.0	6.51	6.8	100.0	6.69	14.8	100.0	6.87
Maximum [3]	13.6	------	------	26.1	------	------	14.5	------	------	26.6	------	------
pH [4]	------	------	8.26	------	------	7.64	------	------	8.03	------	------	7.47

[1] Iron, manganese, molybdenum, and calcium were used at concentrations of 0.08, 0.02, 0.02, and 0.40 mg per liter, respectively.
[2] The extent of growth was estimated by the turbidity of the cultures. The values give the depth of the fixed turbidity standard required to match a 10-mm depth of the culture.
[3] Maximum individual yield of control obtained with 500-cc flasks.
[4] Initial acidity of nutrient solution.

Many of the experiments with azotobacter included tests for ammonia (Nessler), hydroxylamine and nitrite (Griess), and nitrate (diphenylamine), which were performed on the nutrient solution without removal of the bacteria (*11*). Hydroxylamine was oxidized with iodine to nitrite. At both levels of aeration tested, the cultures with free nitrogen gave positive tests for ammonia only (*11*), nor did a trace-element deficiency alter this response. With nitrogen supplied as nitrate, the cultures gave tests for all four constituents at the lower aeration level (50 cc nutrient solution in 125-cc flasks). At the higher aeration level (25 cc nutrient solution in 200-cc flasks), the cultures with nitrate nitrogen gave positive tests for ammonia and unconsumed nitrate, and for hydroxylamine when iron, manganese, or calcium was not added to the solution. Only the minus-calcium cultures gave a positive test for nitrite. No tests, unfortunately, could be made at the optimum aeration level, but it would seem quite

probable that at this level also a graduated deficiency of the trace elements may result in the accumulation of sufficient hydroxylamine in the nitrate solution to give a positive reaction.

DISCUSSION

Inability to duplicate closely the yields of controls in successive experiments has had the effect of diminishing the value of the data. This was overcome to some degree by frequent repetition and check. Under these conditions, of course, it was not possible to more than approximate optimum solutions for growth. Though the cause of these uncontrolled variations is unknown, continued tests with many of the chemical elements failed to identify one of these as the cause. Duplicate cultures, however, gave quite uniform results, and the same was often true for different treatments of the same experiment.

The use of the method of nutrient-solution purification with magnesium or calcium carbonate led to some improvement in results, but less than that obtained with aspergillus. The reason for this probably lies partly in the very small increase in mass of bacteria as compared to fungi for the same quantity of nutrient. On the assumption of even a 200-mg yield per 1.0 g of sucrose, the coefficient of utilization would be only 20 as compared to 50 for aspergillus. It is not surprising, therefore, that the quantities of essential impurities remaining in the nutrient solution after purification, which are still partially effective in the case of aspergillus, are even more effective with rhizobium and azotobacter, especially at low growth levels, in supplying the deficiencies of the elements experimentally omitted. This does not mean, however, that the method of nutrient-solution purification is useless with bacteria but rather that an additional improvement in technique is highly desirable, a fact already known from investigations with aspergillus.

Another factor making for poor deficiency results with the trace elements is the low growth of the controls. Increased growth of control is generally accompanied by improved deficiency results, as has been demonstrated with aspergillus by the writer. This may be accomplished to a considerable extent by the use of greater quantities of bactopeptone with rhizobium and improved aeration with azotobacter, without a further increase in mineral ingredients. It is doubtful, however, that all inorganic constituents in the solutions of table 5 will be present in ample amount when growth factor and oxygen are no longer limiting factors for the respective organisms.

Vigor of culture is best expressed as the maximum bacterial count obtained in these experiments. That for rhizobium was 500,000,000 bacteria per cubic centimeter by actual count. The maxima for azotobacter were estimated at 56,000,000 per cubic centimeter with fixed nitrogen, and 19,000,000 without; improved aeration led to increased counts of 52,000,000 per cubic centimeter without fixed nitrogen and 166,000,000 with fixed nitrogen. Marked increase in growth of rhizobium could be brought about by increased bactopeptone. The quantity of nitrogen fixed by a 50-cc culture in a 125-cc flask of a turbidity of 4.5 mm was too little to be determined by microtitration.

Except for the inability to duplicate successive controls to within a small variation, the data on the trace elements are, on the whole, quite similar to those with aspergillus even at the foregoing nutrition

levels. Omission of an essential element led to a decrease in growth and diminished the rise in acidity accompanying growth. The need of these organisms for iron, manganese, molybdenum, and calcium, even under the suboptimum conditions of these experiments, appears to be specific on the basis of many tests with salts of 77 elements. On the basis of the experience gained in these experiments, the belief was developed that the mineral requirements of the bacteria for growth are, in general, as rigid and inflexible as those for fungi and other forms of life, and that the evidence to the contrary is entirely inadequate, being based on results due to use of impure chemicals.

The evidence for the necessity of a growth factor by rhizobium is based partially on the inability to obtain growth in the absence of this factor by the addition of salts of the chemical elements or by the addition of many organic compounds capable of serving as sources of carbon and nitrogen. The degree of dependence on an accessory growth factor would appear to differ with the strain (*1*, *2*). With the trace of accessory growth factor in the sucrose, *Rhizobium trifolii* No. 532 of L. T. Leonard, which was also tested to a considerable extent, invariably gave larger yields than the No. 205 strain of E. B. Fred and also differed from the latter by giving slight increases in growth with sodium thiosulphate. Moreover, with sufficient decrease in accessory-growth-factor content of the nutrient solution, the No. 532 strain is unable to grow. With this strain, also, the need for an accessory growth factor cannot be avoided by the use of mineral constituents or organic carbon and nitrogen compounds. Furthermore, the use of nitrogen as ammonium salt, nitrate, or asparagine did not preclude the need for an accessory growth factor.

The data afford additional confirmation for the essentiality of coenzyme R [5] for rhizobium (*1*, *2*) and evidence for the existence of another essential growth factor. The tests consisted in the addition to a nutrient solution, containing ample quantities of all mineral constituents, of many organic nitrogen compounds and of salts of 77 chemical elements. These compounds were unable to replace the need by rhizobium for both coenzyme R and the second growth factor ("rhizobiosin") in an unpurified solution. Neither could one of these compounds, totaling more than 140, be substituted for rhizobiosin when added with nutrose to a purified solution. The possibility that an inorganic deficiency is responsible for the results attributed to rhizobiosin is quite slight. The possibility that the addition of 100 mg of magnesium carbonate per liter in the purification resulted in the formation of a soluble toxic substance is likewise remote. The addition of magnesium carbonate to the nutrient solution without subsequent filtration does not necessarily diminish growth and may cause an increase.

The presence of many mineral impurities in the crude growth-factor preparations is shown in the tabulation on page 469. The improvement in results on the trace-element requirements of rhizobium by purification of the bactopeptone preparation has also been shown. It is evident, therefore, that unless precautions are taken the increase in yield brought about by a crude preparation may be due to inorganic constituents as well as growth factors.

[5] According to verbal information from F. E. Allison (*1*, *2*), the accessory growth factor in nutrose is coenzyme R.

The data of table 3 should be interpreted with caution in view of the probable need of two accessory growth factors by rhizobium since the values may depend to a considerable extent on a selective extraction of one or other of the necessary accessories. Though no attempt was made to ascertain the existence of selective absorption by these solvents, the possibility exists.

The solutions used in the culture of azotobacter were of known composition and free from sediment or turbidity. As with aspergillus, purification with magnesium or calcium carbonate gave the best results in tests of trace-element requirements if hot filtration was employed. Hot filtration, however, tended to remove excessive quantities of phosphate and magnesium from the solution, associated perhaps with the absence of ammonium salt. Cold filtration gave better growth of the control, but poorer results on trace-element requirements. As with aspergillus, purification with calcium carbonate seemed better on the whole than that with magnesium carbonate, and the results of deficiency tests with iron were better than those with manganese or molybdenum. Again, as with aspergillus increased growth was usually paralleled by increased acidity. It should not be overlooked, in addition, that though, with increased aeration, the increased growth will probably necessitate higher concentrations of mineral constituents under conditions for maximum growth, the preparation of a simple optimum solution free from turbidity appears to be practical with potassium phosphate, magnesium sulphate, and perhaps sodium or potassium bicarbonate. The marked decreases in yield following nonaddition of molybdenum that have been reported by Bortels (*3*) and Burk and Lineweaver (*5*) have not been duplicated, however. Though the factors responsible have not been determined, it would not be illogical to assume that the trace of molybdenum in the sucrose (about 0.008 mg per 20 g sucrose), found to be present in studies with aspergillus (*15*), plays a large part.

The results obtained with nutrient-solution purification must be realized to be a first trial of the method with bacteria, and subject to improvement with further study. Extraction of the sucrose with 95-percent alcohol after the manner employed for aspergillus should also lead to improved results, particularly with molybdenum. Additional betterment in results with azotobacter should follow at higher aeration levels, with longer growth periods, and through use of strains with relatively low oxygen optima. Another important factor is the freedom from mineral impurities of the accessory growth factors with rhizobium.

SUMMARY

The use of nutrient-solution purification led to a slight improvement in results in studies of the trace-element requirements of *Rhizobium trifolii* Dangeard and *Azotobacter chroococcum* Beij., even at low nutrition levels. The results obtained were quite similar in general to those previously reported for aspergillus. No evidence could be obtained that the need of these bacteria for an essential element is not specific and almost absolute. Evidence was obtained affording additional proof of the essentiality of coenzyme R for growth of rhizobium. The necessity is also indicated of a second accessory growth factor for which the name "rhizobiosin" is proposed.

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U. S. GOVERNMENT PRINTING OFFICE: 1938